THE ROLE OF CHICK CILIARY NEUROTROPHIC FACTOR IN REGULATING CELL DEATH IN CILIARY GANGLION NEURONS

by

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Dedication

To my parents and family,

to Anh-Chi Le,

and to all the members of the Nishi lab.

"Our real teacher has been and still is the embryo-
who is incidently the only teacher that is always right"

- Viktor Hamburger

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List of abbreviations

BDNF	brain-derived neurotrophic factor
CG	ciliary ganglion
chCNTF	chick ciliary neurotrophic factor
CIPE	choroid, iris, and retinal pigmented epithelium
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CNTFRα	ciliary neurotrophic factor receptor alpha
E8	embryonic day 8
GDNF	glial-derived neurotrophic factor
gp130	glycoprotein 130
GPARα	growth promoting activity receptor alpha
FGF	fibroblast growth factor
HIC	hydrophobic interaction column
HPLC	high pressure liquid chromatography
HPLC-RP	high pressure liquid chromatography-reverse phase
IR	immunoreactivity
IL-6	interleukin-6
IL-11	interleukin-11
LIF	leukemia inhibitory factor
LIFRβ	leukemia inhibitory factor receptor beta
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
NT-6	neurotrophin-6
PEI	polyethyleneimine

PNPF	polyornithine binding neurite promoting factor
PNS	peripheral nervous system
rchCNTF	recombinant chick ciliary neurotrophic factor
rCNTF	recombinant rat ciliary neurotrophic factor
rGPA	recombinant growth promoting activity
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid

Abstract

One of the most profound influences targets have upon the neurons that innervate them is the regulation of the level of neuronal cell death that occurs during development. One way in which targets could regulate neuronal survival is through the limited availability of specific trophic factors produced by the targets that the neurons require for survival. The avian ciliary ganglion is one of the best systems to study such neuron-target interactions because of its relative simplicity. Like many neuronal populations, neurons of the ciliary ganglion undergo a period of cell death, whereby approximately half of the original number of neurons present at embryonic day 8 die off by the end of the cell death phase at day 14. The best candidate for a ciliary ganglion target-derived trophic factor is chick ciliary neurotrophic factor (CNTF), also known as growth promoting activity (GPA). Chick CNTF is a potent neurotrophic factor for ciliary ganglion neurons *in vitro*. The studies outlined here extend the characterization of chick CNTF as a target-derived neurotrophic factor and test the hypothesis that chick CNTF regulates CG neuronal survival *in vivo*.

Several steps were taken to test this hypothesis. First, a recombinant form of chCNTF was prepared using two different expression systems and a total of three purification protocols and the purified product characterized on CG neurons in culture. Recombinant chCNTF typically has an ED_{50} of 10-30 pg/ml, and while cultured E8 CG neurons are completely dependent on this factor for survival, E14 and E16 neurons seem largely independent for the need of chicken CNTF or any other trophic factor.

Second, polyclonal and monoclonal antibodies were prepared against recombinant chick CNTF and used to determine if target cells express chick CNTF and if so at what time points in development. Chick CNTF-like immunoreactivity was present in both targets of CG neurons: choroid smooth muscle cells and striated muscle of the ciliary body. In the choroid layer specific immunoreactivity could first be detected by E10 in the choroid layer and increased substantially by the end of the cell death phase. Immunodepletion

experiments with fractionated eye extracts showed that chick CNTF comprises about half of the total trophic activity present in the eye. The level of activity was approximately the same at the beginning and middle of the cell death phase, but increased more than 10-fold by E18.

Third, several different approaches were tried to either elevate or reduce endogenous CNTF levels and then determine the effect it had on CG survival. Expression of chick CNTF through a replication competent retroviral delivery system increased endogenous levels 2.8-fold and a 48% increase in the number of neurons present at E14 was seen. Injection of the recombinant factor, on the other hand, did not significantly enhance neuronal survival. However it could rescue neurons very successfully under lesion conditions. To reduce chCNTF levels, an antisense strategy was used to reduce endogenous chicken CNTF 50-60%. This level of reduction was apparently not sufficient to significantly reduce CG survival *in vivo*. Taken together, these results support the hypothesis that chick CNTF is indeed a target-supplied trophic factor present in limiting amounts that regulates CG neuronal survival.

I. Introduction and Background

1.1. Neuronal cell death in the vertebrate nervous system and the involvement of neurotrophic factors -The neurotrophic hypothesis.

Programmed cell death is a common mechanism used to sculpt, fine tune, and selectively delete subpopulations of cells in biological systems. In the central and peripheral nervous system it is used to match the size of neuronal populations with the target tissues they innervate. The term programmed cell death refers to the fact that RNA and protein synthesis are required to induce cell death[1, Milligan, 1994 #4173, Oppenheim, 1990 #4055, 2, 3]. Thus, such cells are activating a suicide cascade to eliminate themselves. While the level of cell death and timing differs for each population of neurons, typically 50% of the original population is eliminated[4]. The reason for this process is thought to be to match the correct number of neurons with the target cells that are innervated. In invertebrates, such as *C. elegans*, this process is determined genetically, with the level of cell death being programmed such that the same cells always die[5]. In vertebrates, developmental neuronal cell death occurs by an apoptotic mechanism, but a more complex scheme is used to determine which cells live and die.

In vertebrates, where a target tissue is controlled by a large population of neurons rather than a single neuron or a small set of neurons, excess neurons are removed by a target-dependent process. Shortly after neurons have sent processes out to their target fields and have formed immature, but functional synapses on target cells, the neurons become dependent upon their targets for survival[6-9]. Addition of extra targets before cell death has begun results in enhanced cell survival[10, 11], while ablation of some or all of the target tissues results in increased cell death[12-14]. The size of the target field, therefore, regulates the number of neurons that survive. If one makes more target available to one subpopulation of neurons by eliminating a second subpopulation that innervates the same target through axotomy, then additional neurons will survive in the case of the first population[8]. Conversely, if one forces two populations of neurons to innervate the same

target, for example, fewer neurons will survive than if each population had its own separate target field to innervate[15].

The target-dependent process is postulated to be a competition by the neurons for a limiting target-derived molecule. The limitation of the factor would be such that insufficient quantities of the factor are synthesized and made available to the neurons to support all of their survival (see Fig. 1). Those neurons that receive an insufficient amount of the target-supplied signal undergo apoptosis. Note that sensitivity to the factor might not be a linear relationship, i.e. a doubling in the level of factor may not result in a doubling of the amount of neuronal survival. In its simplest form, the neurotrophic hypothesis predicts that a given cell type will begin to synthesize, process (if necessary), and secrete a particular neurotrophic factor some time before or just after first being innervated. An important consideration is that competition for a target-derived factor is not a race to send processes out to the target in order to sequester a limited molecule, for nearly every cell has made functional synapses at the targets before cell death has begun[8]. Once released from target cells, such neurotrophic factors will bind to high affinity receptors present at the nerve terminals and will lead to intracellular signaling and retrograde transport of the factor back to the cell soma, in a process that ultimately (though still largely undefined) overrides programmed cell death.

While much has been learned descriptively about cell death and some of the factors that may be involved in regulating how many neurons survive, little is known about how neurotrophic factors accomplish this process. How are their levels regulated so precisely that just the right amount of neurons survive? What intracellular signals are invoked/overridden to promote neuronal survival? Much of the work in this area in the past 40 years has centered on identifying and studying individual neurotrophic factors produced by target tissues.

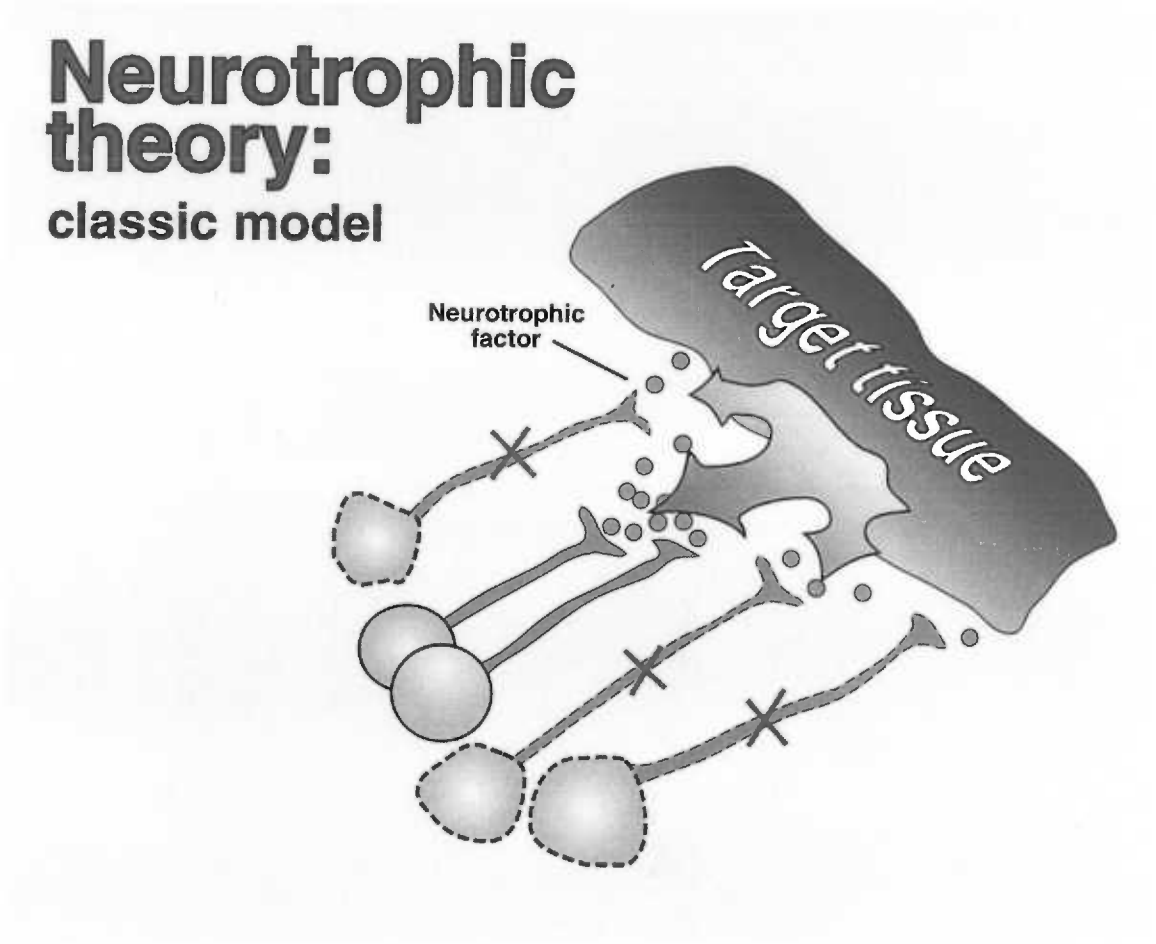

Figure 1. **Classic model of the neurotrophic hypothesis as it was first described.** Shortly after neurons innervate their targets, they become dependent upon the targets for survival. If the targets are removed at a time before cell death begins, most of the neurons die off. If additional target tissue is grafted on, more neurons are promoted to survive. The neurotrophic hypothesis states that a specific trophic factor made by the target in limiting quantities is what regulates cell death. Under this model, neurons compete for the factor, and those that successfully obtain a sufficient quantity of factor survive and continue to develop, whereas those that do not die by apoptosis.

1.2. NGF as the prototypical example of a target-derived neurotrophic factor.

Of the more than a dozen proteins identified thus far with neurotrophic activity, only a few can be classified as true target-derived neurotrophic factors. Rigid criteria have been established for the classification of molecules as true target-derived neurotrophic factors: first, such molecules must act *in vitro* to support long term neuronal survival on a defined neuronal population; second, the factor must be expressed at very low levels by the target cells the neurons innervate during the period when cell death is occurring; third, high affinity receptors for the factor must be expressed by the neuronal population *in vivo* at this same time window of development; fourth, the factor must be secreted by the target cells; and fifth, elevating endogenous levels of the factor must be able to rescue additional neurons from cell death *in vivo,* and reduction of endogenous levels must reduce neuronal survival.

The identification and characterization of NGF as a target-derived molecule for sympathetic and dorsal root ganglia neurons in chick embryos was not a straightforward procedure. What began as a convenient substitute for limb grafts[16] led to a remarkable finding that a mouse tumor could promote dramatic neurite outgrowth and innervation of the tumor along with pronounced elevation in the number of neurons in dorsal root and especially sympathetic ganglia[17]. A similar effect in increased neuronal survival could be seen even if the sarcoma was grown on top of the chorioallantoic membrane. Therefore, whatever the sarcoma was producing was a secreted factor that could exert its effects at a distance in the absence of innervation. In an effort to characterize the class of molecules that might account for this effect, snake venom was used for its high phosphodiesterase activity. Surprisingly, snake venom also had NGF-like activity, as did extracts from the mouse submaxillary salivary gland- the mouse equivalent of a snake venom gland[18, 19]. Fortunately, and for reasons still unknown, the male mouse salivary gland contains high levels of NGF and allowed purification of the molecule using an *in vitro* neurite outgrowth

assay to follow in the purification[19]. In 1969, NGF was purified and its sequence deduced [20] and it represented the first purified protein with neurotrophic activity.

The current evidence for NGF supporting sympathetic neurons in this capacity is very strong: NGF is a potent neurotrophic factor for sympathetic neurons *in vitro*[21, 22]; NGF is expressed in the targets of sympathetic neurons and its levels correlate with innervation of sympathetic fibers[23, 24]; addition of exogenous NGF or a tumor-secreting NGF enhances sympathetic survival[25]; overexpression of NGF in skin results in an increased number of sympathetic neurons[26]; systemic administration of anti-NGF blocking antibodies reduces sympathetic neuronal[27]; mice with a null mutation in the high affinity NGF receptor, TrkA (which localizes to sympathetic neurons[28]), also show a large reduction in the number of sympathetic neurons[29, 30].

This raises a key question- if NGF is such a good example of a target-derived neurotrophic factor that regulates cell death in sympathetic neurons, why study other trophic factors? For one reason, at the time NGF was first discovered and characterized, it was not known if this was simply the first example of what may be many neurotrophic factors in existence that regulate cell death throughout the nervous system, or a unique factor that plays a special role only for sympathetic neurons. Furthermore, NGF clearly does not support all neuronal populations, including some of those of the greatest interest in the study of neurodegenerative diseases. The idea that not all factors may work the same or that all neuronal populations that are dependent upon NGF will respond in the same fashion, is another reason for examining trophic factors aside from NGF and for looking at populations other than sympathetic neurons. Finally, the ultimate goal is not to simply identify target-derived molecules, but to discern *how* they regulate neuronal cell death. NGF was the first factor discovered, and one that clearly plays a role in at least one population of neurons, but that does not necessarily make it the best factor for elucidating trophic factor regulation, availability, neuronal competition, how neurons know which factor to respond to, and how cell death is prevented on a molecular level.

These examples reveal the inherent problem of studying neurotrophic factors *in vivo*- the complexity of neurotrophic dependency. To be more accurate, neurotrophic factor research is as much a study of neuron-target interactions as it is the study of specific biological agents and how they promote survival. The neurotrophic theory is typically represented as a static model, whereby a differentiated neuronal population becomes dependent upon a trophic factor provided by a mature target. The situation is much more complex in reality. In some neuronal populations, cell birth and cell death are occurring simultaneously, the axons that project to the target continue to branch, the synapses become more specialized, and the target itself is usually growing and becoming more differentiated. Trophic factor levels often change during this time as does receptor expression and multiple factors may be involved. How trophic factor availability is regulated during this time period of development such that just the right amount of neurons survive is still unclear.

1.3. The ciliary ganglion as a model for studying neurotrophic dependency and neuron-target interactions. One system that lends itself well to the study of neuronal survival and specific neuron-target interactions is the avian ciliary ganglion (CG). The CG is a parasympathetic autonomic ganglion responsible for both lens accommodation and pupillary light reflex[31]. In birds, it helps regulate blood flow-through the eye in choroid arteries[32]. A large amount of information exists on the physiology, histology at both the light and electron microscopy level, and neurochemistry of these two populations. Although the rodent CG consists of only a few hundred neurons that are dispersed along the optic nerve, avian, rabbit, monkey, and human CG are more numerously populated and well defined[33-36]. The ciliary ganglion undergoes a period of cell death beginning at about E8 and is largely complete by E14, at which time roughly half of the neurons have died off. The ciliary ganglion has several distinct features which are advantageous for studying neuron-target interactions. First, the ciliary ganglion can be readily isolated from E6 through adult chick embryos, and by E8 the neurons become completely dependent upon

trophic support *in vitro*. Second, the CG contains only two populations of neurons, choroid neurons and ciliary neurons (Fig. 2). Third, each population exclusively innervates distinct targets in the eye. CG choroid neurons innervate smooth muscle cells surrounding arteries within the choroid layer of the eye while the larger CG ciliary neurons innervate the constrictor muscle in the iris and muscle fibers in the ciliary body. Fourth, nearly 100% of the neurons can be maintained *in vitro* by either a crude eye extract or purified trophic factors. Fifth, the CG represents a relatively synchronized population, with the ganglion being numerically complete by E6, 2 days before cell death begins (Fig. 3). The easy isolation of such a defined population that is synchronized led a number of groups to use this as a system for screening tissues for neurotrophic activity, and other groups to search for a true chick ciliary ganglion neurotrophic factor.

1.4. The search for CNTF. The identification and isolation of a neurotrophic factor that supported the survival of parasympathetic neurons from the ciliary ganglion (CG) was actively pursued as part of an effort to identify new neurotrophic factors for the following reasons: it had been well established by Landmesser and Pilar [37, 38] that CG neurons proceeded through a period of target-dependent cell death, and CG neuron survival *in vitro* was insensitive to nerve growth factor (NGF) [39], but dependent upon another, immunologically distinct trophic molecule that was likely to be a novel neurotrophic factor. At the time of this discovery, NGF was the only known neurotrophic factor and the idea that CG motor neurons could be used to screen for possible spinal cord motor neuron trophic factors led to considerable enthusiasm in the field. Neurotrophic activity for CG neurons *in vitro* was first identified in heart-conditioned medium[39-42], and then in embryonic tissue extracts[42-45] (see Fig. 4). Some of this activity was characterized as polyornithine binding neurite promoting factor (PNPF), which later was found to be laminin[46]. In 1979 , the chicken eye was identified as the richest source of neurotrophic activity and the highest specific activity was found in a dissected preparation containing the

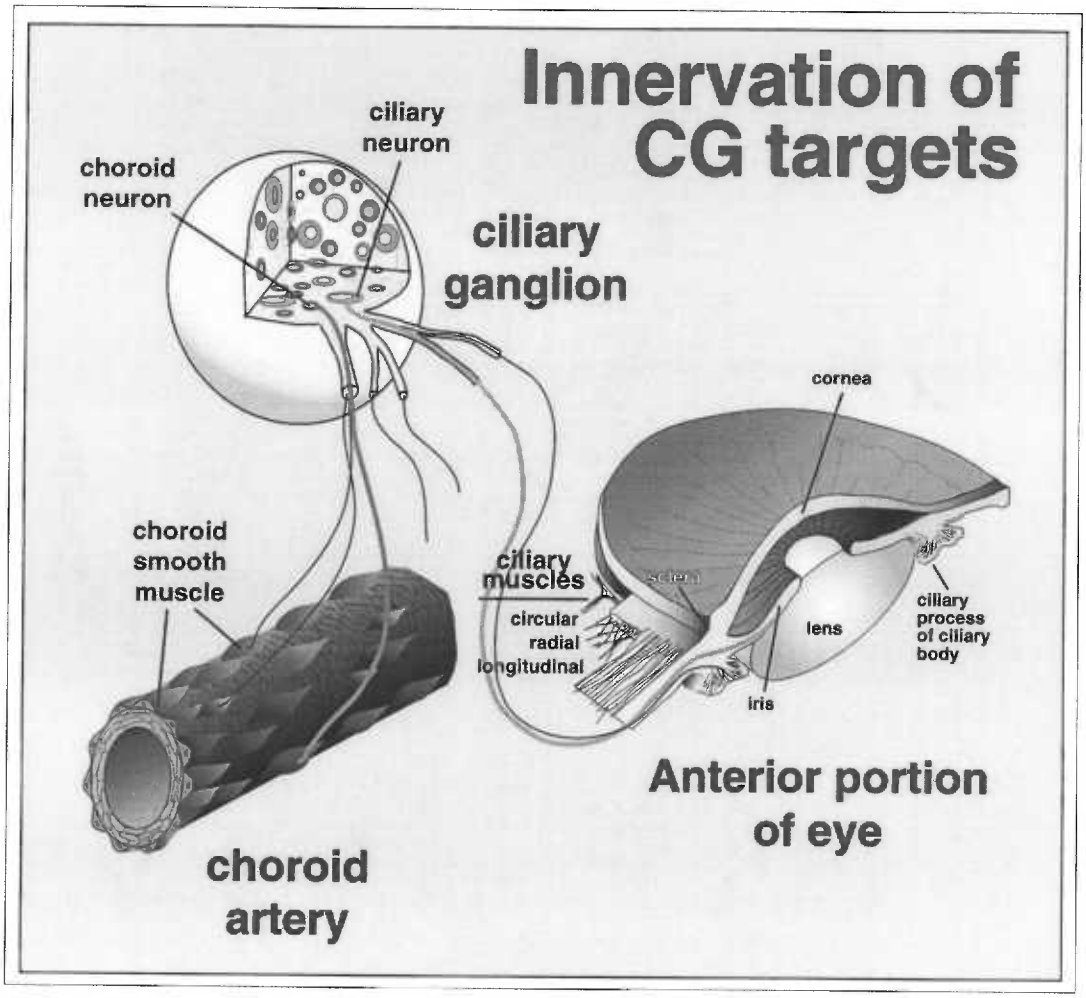

Figure 2. **Targets of the ciliary ganglion.** The ciliary ganglion contains only two populations of neurons: ciliary neurons that exclusively innervate striated muscle cells of the ciliary body and sphincter muscle cells of the iris and choroid neurons that innervate smooth muscle cells surrounding choroid arteries. The ganglion contains roughly equal populations of both neuronal types, and these neurons become target-dependent at the same time.

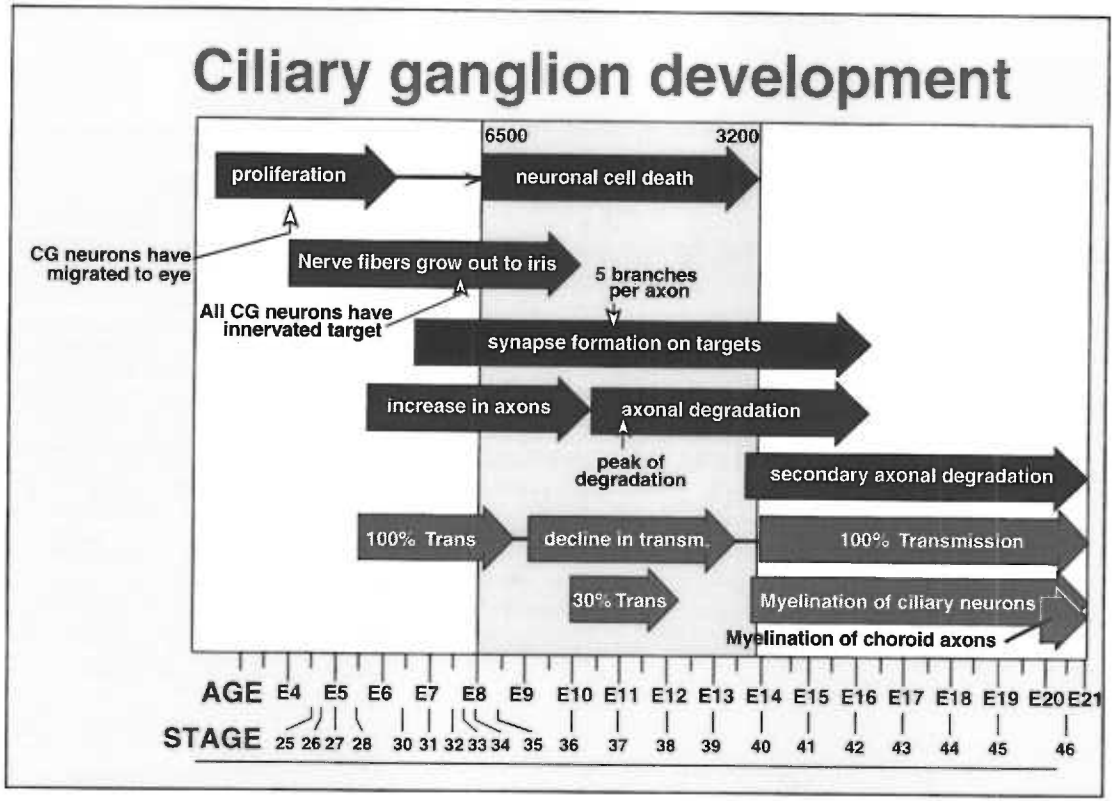

Figure 3. **Time-line showing major points in CG development.** Before cell death has begun in the ganglion, neuronal proliferation has ceased, processes have been sent out to the targets from both populations of neurons, and immature synapses have formed. During cell death, the number of axonal branches and synapses changes, as does synaptic transmission through the ganglion. The reduction of CG neurons folows a sigmoidal curve until E14, when the cell death phase is largely over and roughly half of the neurons have died off. After the cell death phase is completed, additional axons degrade, the number of synaptic branches continues to change, and myelination of ciliary neurons and choroid axons begin.

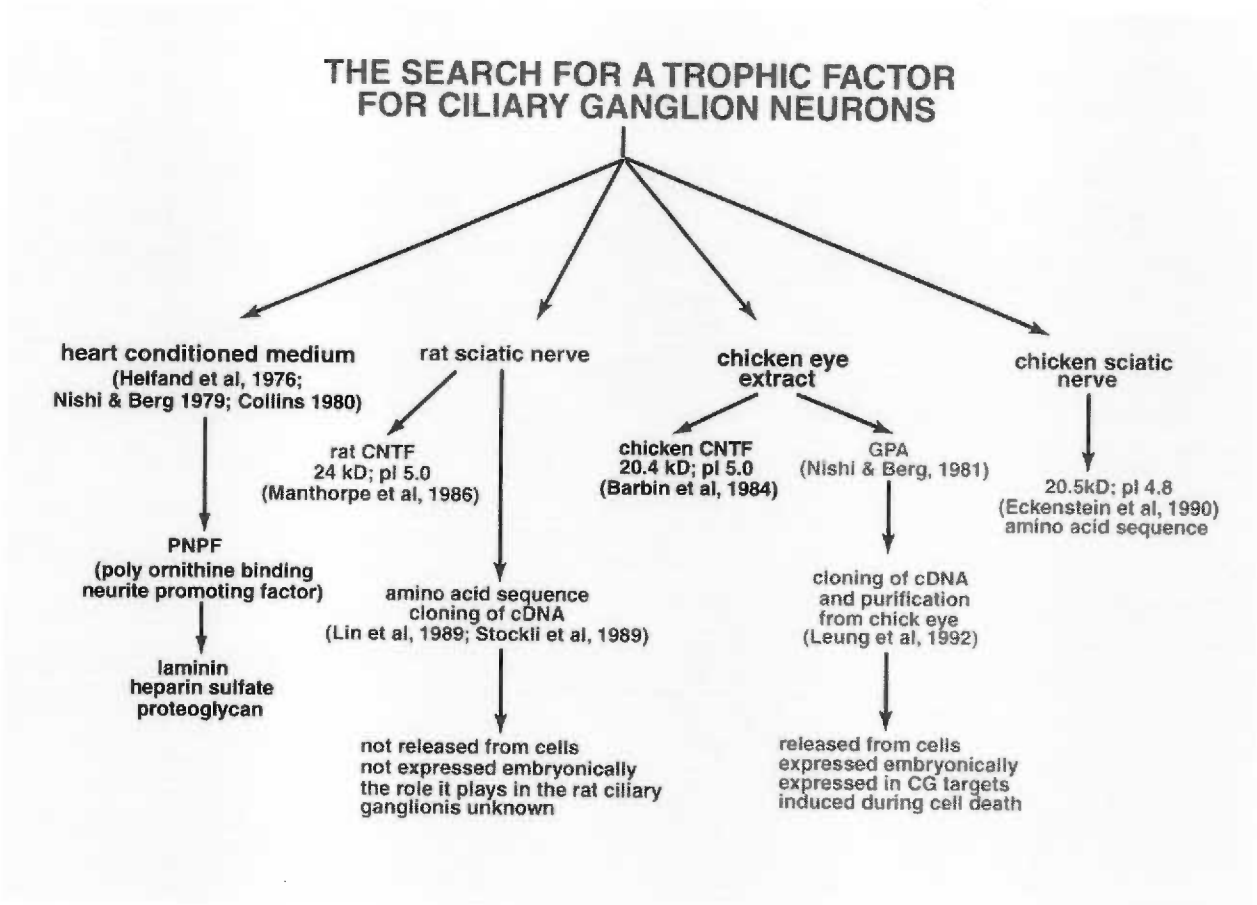

Figure 4. **A history of ciliary ganglion trophic factor purification and cloning.** At a time when NGF was the only known neurotrophic factor, the ciliary ganglion was an attractive system to search for a cholinergic motoneuron trophic factor, as well as one of the few populations of neurons that could be grown in culture with good viability. Many tissue extracts and conditioned media were tested for activity, but purification of CNTF was ultimatelyattempted from heart-conditioned medium, rabbit sciatic nerve, and embryonic chick eye.

choroid, iris, and retinal pigmented epithelium (CIPE)[47]. CIPE contains all the target tissues innervated by CG neurons (vasculature of the choroid and muscles of the iris and ciliary body). The trophic activity was purified 400-fold from the CIPE and named 'ciliary neuronotrophic factor' (CNTF)[48]. In parallel with these studies, soluble extracts from embryonic day 17 chick eyes were fractionated over a gel filtration column and found to contain a 20-25kDa activity which supported growth in size of CG neurons[49]. The fractions containing this trophic activity were pooled and named "growth promoting activity" (GPA).

Shortly after the purification of CNTF from chick eyes, Varon and colleagues also reported the isolation of CNTF from rat sciatic nerve[50]. Disappointingly, no amino acid sequence information could be obtained from either the chick or rat CNTFs reported above. CNTF was purified to homogeneity from rat[51], rabbit[52], and chicken sciatic nerve[53]. The CNTF purified from chicken was called GPA because of the bioassay used to characterize its activity[49]. Partial amino acid sequence of all three neurotrophic factors was obtained from peptide fragments because the N-terminal was blocked. A full length cDNA for rabbit CNTF was cloned from sciatic nerve[52],while rat CNTF was cloned from astrocytes[51]. Despite numerous attempts, chicken CNTF could not be cloned by homology to rat and rabbit CNTFs; rather, the sequence was determined only after GPA was cloned from an embryonic day 15 chicken eye cDNA library using oligonucleotides based upon the partial amino acid sequence of purified GPA[54]. Mouse[55] and human CNTF[56] have also been cloned and their genomic structures identified [57, 58].

1.5. Goals of this thesis.

At the time this project began, NGF was the only trophic factor that had been tested and shown to meet all of the criteria specified by the neurotrophic theory. Since a multitude of factors have been identified, most of which have not been fully tested as target-derived trophic factors, BDNF and NT-3[59, 60] are two trophic factors in the same family as NGF

that have recently been shown to meet the criteria for target-derived molecules for specific neuronal populations. BDNF and NT-3 shown to support a variety of neruonal populations *in vitro* [61-64] and mice transgenic for null mutations in these genes show severe neuronal deficits as do null mutations of their high affinity receptors[59, 60]. Most recently, mounting evidence suggests that a distant member of the TGF-β family, glial-derived growth factor (GDNF), is a trophic factor for enteric neurons[65-67]. Recent studies have also ruled out the role of certain factors in regulating populations of neurons previously shown to meet many of the criteria of a target-derived molecule, most notably, FGF-5[68] in the case of spinal cord motor neurons, and GDNF as a factor that regulates survival of dopaminergic neurons of the substantia nigra [65, 66].

This project was designed to test the hypothesis that chick CNTF is a target-derived trophic factor that regulates neuronal cell death in the ciliary ganglion. To establish chCNTF as a target-derived trophic factor will require satisfying all the criteria of the neurotrophic hypothesis as has been shown for NGF. It is important to clarify at this point that mammalian CNTF was already ruled out as such a factor in general[69]. This is because mammalian CNTF lacks any identifiable signal sequence[51, 52]; it is expressed at highest levels in sciatic and optic nerve[70, 71],which of course are not targets at all; high the levels found in these tissues is inconsistent with it being limiting[72]; and it is not expressed during development[71] (however, see Ip et. al., 1995)[73] when the majority of cell death is occurring.

The project itself was divided up into three phases, each designed to test different criteria for chCNTF being a target-derived trophic factor. In the first phase a recombinant form of chCNTF was prepared and used to establish that the molecule purified from sciatic nerve and embryonic eye is indeed responsible for the neurotrophic activity. The factor was compared to rat CNTF on CG cultures and used to determine some of the trophic requirements CG neurons have *in vitro*. The factor was also used to prepare polyclonal and monoclonal antibodies for use in later stages of the project, such as demonstrating

secretion of chCNTF from cultured choroid smooth muscle cells. In the second phase, the timing and distribution of chCNTF in CG targets was evaluated. Using immunocytochemisty the presence of chCNTF in choroid smooth muscle cells and striated ciliary muscle was examined. Using a combination of quantitative bioassays and immunocytochemical techniques, the levels of chCNTF in the eye was also evaluated, not only from the standpoint of relative levels of expression during development, but also to determine the relative levels of chCNTF with respect to the total amount of CG trophic activity present in the eye. In the third phase I set out to experimentally manipulate endogenous chCNTF levels using a variety of techniques. Strategies were designed to either elevate levels *in vivo* above the endogenous level, or to reduce the levels through a partial block.

Results

Chapter 1

Expression of a chicken ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell death phase

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note: the original manuscript refers to GPA, whose name has been updated to chCNTF and was hereby substituted throughout the original manuscript.

Abstract

Ciliary ganglion (CG) neurons, like other neuronal populations, become dependent upon their targets for survival during development. We have previously purified and cloned growth promoting activity (GPA), a secreted ciliary neurotrophic factor. We report here the expression and purification of a highly active form of recombinant GPA, the preparation of GPA-specific polyclonal and monoclonal antibodies, and the use of these antibodies to investigate the cellular location and timing of GPA expression in tissues innervated by CG neurons. Virtually all of the trophic activity in extracts of embryonic eyes could be depleted by GPA-specific antibodies. GPA-like immunoreactivity was found in both targets of the CG, the arterial vasculature of the choroid layer and the ciliary body of the eye. In the choroid layer, GPA was localized to smooth muscle cells surrounding the choroid arteries. Staining in the choroid layer was first detectable at E10, or about 2 days after cell death has begun in the ganglion, then increases in intensity through E19. Quantification of trophic activity from whole eye extracts at various ages showed a small increase in activity observed between E9 and E12, and at least a 10-fold increase between E12 and E18. The presence of GPA protein in targets cells of CG neurons during the specific time of development when these neurons have become dependent upon targets for neuronal survival supports the hypothesis that GPA is a true target-derived neurotrophic factor that regulates cell death of CG neurons.

Introduction

Neuronal cell death plays a crucial role during development to sculpt neuronal cell number [4]. The degree of change in cell number that occurs is regulated by the tissues that are innervated by the neurons undergoing death; that is, removal of the target exacerbates cell death, whereas addition of more target reduces cell death. A significant contribution to the understanding of neuronal cell death has been the discovery that neuronal cell survival *in vivo* is dependent upon a neurotrophic factor produced by the target tissue. Blocking the action of nerve growth factor (NGF) *in vivo*, for example, causes virtually all sympathetic neurons to be eliminated. If cell death is regulated by the availability of trophic factor, then the addition of exogenous factor during the period of death should rescue neurons. Such experiments have been difficult to analyze in sympathetic and sensory ganglia because the period of cell death is extensive, and proliferating and differentiating neurons are intermixed with dying neurons. Thus, although the addition of NGF was found to decrease the number of pyknotic nuclei, one cannot rule out an effect on proliferation or differentiation of neurons from precursor cells[74, 75].

The avian ciliary ganglion has key advantages for investigating the interaction between neurons and target tissues in detail. The ciliary ganglion is parasympathetic and composed of only two types of neurons, ciliary and choroid[35]. The survival and differentiation of both types of neurons can be monitored throughout development. They withdraw from the mitotic cycle at E5 prior to the time that they undergo target-dependent cell death between E9 and E13[37, 38]. This period of cell death is shorter than that observed in other ganglia because of the relative synchrony of development between the two cell types. The target tissues of the ciliary ganglion are all contained within the eye: ciliary neurons innervate striated muscle of the iris and ciliary body; choroid neurons innervate smooth muscle of the vasculature in the choroid layer[76]. Both of these targets can be readily dissected and separately manipulated. For example, substances injected into the

anterior chamber of the eye have access to the iris/ciliary body but are physically separated from the posterior chamber where the choroid is located. Choroid neurons can be distinguished from ciliary neurons by the expression of somatostatin immunoreactivity[77, 78]. Finally, cells from the target tissues as well as both neuronal populations of the CG neurons can be readily maintained in cell culture[43, 45, 79]. Thus, the effects of specific molecules on neurons can be monitored, and hypotheses regarding their action can be tested.

Two molecules have been isolated and cloned on the basis of their ability to support CG neuron survival in cell culture. Ciliary neurotrophic factor (CNTF) was purified and cloned from rat and rabbit sciatic nerve[51, 52]. The full length cDNA for rat CNTF lacks an N-terminal signal peptide and is not secreted from transiently transfected cells. Hence, CNTF is not thought to be a true target-derived trophic factor in the rat[69]. Since antibodies and cDNA probes for rat CNTF were unable to cross-react with chick CNTF, we purified and cloned a chicken molecule which was initially identified as growth promoting activity (GPA[49]). GPA shares 48% amino acid identity with rat CNTF and also lacks an N-terminal signal sequence. However, GPA is secreted from transfected cells and its mRNA is expressed in the eye during embryonic development[54].

Our hypothesis is that GPA is a target-derived trophic factor for chick ciliary ganglion neurons *in vivo*. In order to extend our studies, it was necessary to express and purify a highly active recombinant GPA which was then used to make GPA specific polyclonal and monoclonal antibodies. The antibodies were used to test whether the neurotrophic activity in CG targets was GPA by performing immunodepletion experiments. Immunocytochemistry was also used to determine whether the cell-specific distribution and developmental expression of GPA protein was consistent with our hypothesis. These studies now set the stage for the future *in vivo* manipulations that will be necessary to rigorously test our hypothesis.

Materials and Methods

Expression and purification of recombinant GPA. Two different purification protocols (A and B) were devised to purify GPA. In both protocols, the coding region for GPA was inserted into a low expression procaryotic vector under the direction of a tryptophan promoter in order to increase the chance of producing recombinant chicken GPA (rGPA) in a soluble form. For protocol A, rGPA was induced by incubating log phase cultures in the presence of 25 mM indole acrylic acid (Sigma, S. Louis, MO) overnight at 37°C with vigorous shaking. Cultures of the expressing strain were prepared by inoculating 8 liters of minimal medium (supplemented with 15 μg/ml tetracylcine and 0.5% casaamino acids) with a log phase culture. Cultures were incubated for 1 hour, then induced with 1 mM indole acrylic and incubated overnight with vigorous shaking. Cells were then harvested by centrifugation, treated with 1 mg/ml lysozyme for 30 minutes at 37°C, then lysed by sonication for 4 minutes in the presence of a cocktail of protease inhibitors. The cell lysate was centrifuged at 43,000 x g at 4°C for 60 minutes, and the supernatant dialyzed against 20 volumes of dialysis buffer with 3 changes of buffer. The dialysis material was centrifuged at 41,000 x g at 4°C for 60 minutes and the supernatant applied to a DEAE-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl, pH 7.4. A 0 to 1M NaCl gradient was applied and the peak rGPA fraction determined by a short term ciliary culture survival assay[53]. Recombinant GPA eluted at about 225 mM NaCl from DEAE Sepharose, as did the native form of GPA[53]. The peak fraction was concentrated to less than 2 mls by ultrafiltration, combined with SDS-PAGE sample loading buffer and run on an 11 cm preparative SDS-PAGE tube gel for a total of 36 hours at 25°C. Fractions were assayed for biological activity and the peak fractions pooled and concentrated by ultrafiltration. SDS was removed from the final product by two passes over a Sephadex G-10 column (Sigma).

For protocol B, total cellular lysate was passed over a larger DEAE column under the same conditions as in protocol A, and the peak fraction concentrated and applied to a Sephacryl S300HR column (Pharmacia). Recombinant GPA was detected in multiple peaks. Recombinant GPA containing fractions were bioassayed, the most active fractions pooled, adjusted to 2M $(NH_4)_2SO_4$, and passed over a Toyopearl hydrophobic interaction column (HIC). Recombinant GPA did not bind to the column and came through in the flowthrough. The HIC flowthrough was adjusted to 0.1% trifluoroacetic acid (TFA) and run over a Toyopearl reverse phase column, where it again eluted as multiple peaks. Each fraction was bioassayed and the peak fractions pooled into three groups based on the level of biological activity (pools A, B, and C). Only the pool of highest biological activity, pool A, was used in the experiments outlined here.

Expression and purification of recombinant rat CNTF. Recombinant rat CNTF (rCNTF) was expressed in the same vector as that for GPA and was purified by a variation of protocol B. After DEAE chromatography, rCNTF containing fractions were pooled, concentrated by ultrafiltration and applied to a Sephacryl S200 column. Fractions containing rCNTF, as determined by SDS-PAGE, were pooled and concentrated, adjusted to 0.2% TFA and run on a Vydac C4 HPLC reverse phase column. A 15% to 95% acetonitrile gradient was applied to the column and rCNTF eluted as a sharp peak. The final product was tested using E8 CG neuronal survival assays.

GPA bioassay. Short term (2-5 days) CG neuronal survival assays were performed according to Eckenstein, et al., 1990[53], using E8 ciliary ganglia. Neuronal survival was determined by counting the number of large, phase-bright cells with processes greater than 3 cell diameters in 72 hour cultures.

Culture methods. E8 (St. 34) ciliary ganglia were removed and dissociated as previously described [53] and cultured in Eagles Minimal Essential Medium (MEM, Gibco-BRL, Grand Island, NY) containing 10% (v/v) heat-inactivated horse serum (Gibco-BRL) with 50 U/ml penicillin, 50 µg/ml streptomycin, and 2mM glutamine.

Production of GPA specific polyclonal and monoclonal antibodies. 50 µg of rGPA purified by protocol A was diluted into PBS and injected into the lymph nodes of New Zealand white rabbits. Rabbits were given three boosts at three week intervals and their titer monitored by Western blot analysis against a crude bacterial lysate of induced cultures. Monoclonal antibodies were prepared according to Kohler and Milstein, 1975[80]. Briefly, four Balb/C mice were given a total of five 25 µg injections of rGPA (protocol A and B) over a four month period. Mice with positive titers for rGPA on dot blots were sacrificed by asphyxiation with CO_2, their spleens removed and the splenocytes were fused with NS-1 myeloma cells using polyethylene glycol (PEG). The resulting hybridomas were cultured in a modified Leibowitz L-15 medium[81] containing 15% (v/v) prescreened fetal bovine serum (Whittaker), 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and HAT (0.1 mM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine). After 6 days, the HAT was replaced with HT (0.1mM hypoxanthine and 16 µM thymidine). The surviving clones were screened initially by dot blot analysis against rGPA. Specificity to rGPA was determined by Western blot against both partially pure (DEAE peak) and pure rGPA. Positive antibody preparations were tested for cross-reactivity to CNTF on Western blots against recombinant rat CNTF using equal amounts of CNTF and GPA.

Western blot analysis and immunoprecipitations. All samples were run on 16% SDS-PAGE gels in beta-mercaptoethanol-containing sample buffer, transferred to nitrocellulose (Schleicher and Schuell, Keene, NA) or PVDF (MSI, Westboro, MA) for 30 min. at 150 mA using a semidry blotting apparatus, and blocked for at least 24 hours at 4°C in 10%

horse serum/ 2% powdered milk/0.1% Triton-X100 in phosphate buffered saline (PBS). Samples contained 100 ng of either a crude bacterial lysate of rGPA, purified rGPA, or purified recombinant rat CNTF. The blots were incubated with primary antibodies diluted in 10% horse serum, 0.1% Triton-X100 for 60 minutes at 25°C, washed 3 times in 0.5 M NaCl/ 0.1% Triton-X100/PBS and 3 times in PBS, then incubated for 60 minutes with a biotinylated secondary antibody (Vector Labs), washed, and incubated with alkaline phosphatase coupled avidin (Gibco-BRL) for an additional 60 minutes. Blots were developed using NBT/BCIP as a substrate. In cases where immunoprecipitation was employed, samples were incubated with either rabbit anti-GPA polyclonal antibodies bound to protein A-Sepharose beads or normal rabbit serum (NRS) prebound to protein-A Sepharose (Sepharose). Samples were incubated overnight at 4°C, centrifuged, and the supernatants stored at 4°C until bioassayed.

Immunocytochemistry. The choroid layer can be isolated from the rest of the eye by dissection beginning at about E7. By peeling apart the three eye layers (Fig. 4b), the choroid layer can be clearly separated from the two other layers, and given its thin and transparent nature can be stained as whole tissue. For floating preparations of choroid layers, E7-E19 eyes were removed from embryos, cleared of extraneous tissue, and an X-shaped incision made at the back of the eye. The vitreous was then pulled away and the neural retinal layer removed (with the exception of the pigmented epithelium that adheres to the choroid layer in embryos younger than E15). Pigmented epithelium was removed using cotton swabs leaving only the clear choroid and sclera layers of the eye. Tissues were fixed for 20 minutes in 1% paraformaldehyde and 15% picric acid for 30-60 minutes at room temperature with gentle shaking, and the fixative removed by five washes in PBS. After fixation, the sclera was removed with a pair of fine forceps and the isolated choroid layers incubated for at least 24 hours in 10% horse serum, 0.5% Tween, 0.2% azide in PBS (blocking solution). For sciatic nerve, adult chicken sciatic nerves were removed,

cleared of extraneous tissue, and fixed overnight at 4°C with gentle shaking in freshly prepared 4% paraformaldehyde. The fixative was removed by five washes with PBS, and the nerves incubated each in 15% sucrose/PBS, 30% sucrose/PBS, 50% Tissue-Tek (OCT) (Miles, Inc., Elkhart, IN), and 100% Tissue-Tek. 20 μm cryostat sections were made in both longitudinal and cross section orientations and the tissues incubated in blocking solution for at least one week at 4°C.

Whole eye tissues and cryostat sections were stained by a double peroxidase-antiperoxidase (PAP) staining procedure using diaminobenzidine as a substrate with nickel enhancement. Cryostat sections were stained as floating sections. Briefly, cryostat sections were preincubated overnight with blocking solution, then incubated for 60 minutes in primary antibodies at room temperature followed by a 30 minute incubation in H_2O_2 to inactivate endogenous peroxidases. Sections were then incubated with a 1:400 dilution of either goat anti-mouse or goat anti-rabbit (Sternberger Monoclonals, Baltimore, MD) for one hour, followed by a 1:400 dilution of rabbit or mouse PAP (Sternberger Monoclonals) for one hour. Sections were then incubated with another round of secondary and PAP complex and incubated with diaminobenzidine substrate for 8 minutes. All steps were followed by washing 6 times in 0.5% Triton-X100, 5% horse serum in PBS. Polyclonal anti-GPA and CNTF were used at a 1:500 dilution. As a means of blocking specific staining, anti-GPA polyclonal serum was passed 3 times over a 1 ml rGPA-affigel column prepared using reverse phase purified rGPA, pool A, crosslinked to Affigel-15 (Biorad, Hercules, CA). The flowthrough of the column was aliquoted and stored at -80°C until needed. For eye tissues, choroid layers were stained as floating tissues in 0.4 ml solution per well in 48-well plates. Immunolocalization of GPA was most successful with portions of whole choroid layer stained floating. To compensate for the thickness of the tissues, incubations were increased as follows: primary antibodies 12 -18 hours, secondary and PAP complexes 12 hours each, wash times 20-30 minutes per wash, 3-4 washes per solution. Primary antibodies were incubated at room temperature, while all subsequent

steps were performed at 4°C, with the exception of the DAB substrate. NiCl was also used to enhance the intensity of staining. Primary antibodies were used at 1:800 and anti-GPA monoclonal antibody 13p at 1:4. Ciliary body and iris were treated in a similar way to choroid, except pigmented cells of the iris and ciliary processes were partially removed with a cotton swab before antibody incubation. E12 was selected as a time point because it is during CG cell death when GPA should be present and the choroid layer is well defined. For colocalization of GPA and SMSA, TRITC-labeled anti-SMSA antibodies (Sigma) and rabbit anti-GPA sera (both used at 1:500) were incubated for 4 hours with floating choroid layers at 25°C. Samples were then incubated with FITC-labeled goat anti-rabbit secondary antibodies (1:500) for 2 hours. Some samples were incubated with TRITC-SMSA alone to check for specificity of fluorescein and rhodamine filters. All photomicrographs were scanned on a color flatbed scanner and adjusted to equal brightness/contrast in Adobe Photoshop, then printed on a dye-sublimation printer as composites.

Preparation of target tissue extracts. E8-E18 eyes were isolated and cleared of extraneous tissue, including the optic nerve, CG, and extraocular muscles, then the vitreous removed. Tissues were immediately frozen in liquid nitrogen and stored at -80°C prior to processing. An extract was prepared by grinding the thawed tissue in a Tekmar homogenizer in 10 mM NaMOPS, 5 mM EDTA, pH 7.2 in a cocktail of protease inhibitors, followed by sonication with a Cole-Parmer 4710 probe type sonicator for 2 minutes on ice. All subsequent steps were performed at 4°C. The extract was centrifuged at 26,000 x g for 30 minutes at 4°C and the supernatant dialyzed overnight with 2 changes of buffers. The extract was centrifuged again at 26,000 x g for 60 minutes and the supernatant passed over a DEAE-Sepharose column pre-equilibrated with 10 mM NaMOPS, pH 7.2. A step gradient of 0.15, 0.3, and 1M was applied and the 0.3 M fraction applied to a heparin-agarose column (Biorad). The flowthrough was divided into aliquots with one aliquot being incubated with anti-GPA polyclonal sera prebound to protein-A-Sepharose beads and another to NRS-

protein-A-Sepharose. Other aliquots were boiled for 30 minutes, incubated with 1 mg/ml proteinease K (final concentration) (Boehringer Mannheim, Indianapolis, IN) for 30 minutes at 37°C, or left untreated. All samples were 0.22 μm filter sterilized prior to use in the bioassay.

Results

Production and characterization of GPA-specific antibodies.

Recombinant GPA (rGPA) expressed in a procaryotic expression vector supported survival of E8 CG neurons (Fig. 1). Both the SDS-PAGE and reverse-phase purification protocols yielded about the same purity rGPA; however, SDS-PAGE purified GPA had a higher specific activity. GPA had a tendency to aggregate during purification and inclusion of detergent increased its biological activity. The ED_{50} of our best preparation of rGPA was approximately 1 pg/ml. Compared to purified sciatic nerve GPA, rGPA was more active on E8 CG. E8 CG neurons cultured in the presence of rGPA had large phase bright cell bodies with many processes and could be maintained in culture for at least 3 weeks.

Polyclonal and monoclonal antibodies were prepared using both preparations of rGPA described in Materials and Methods. The specificity of these antibodies was verified on Western blots against both rGPA and rCNTF (Fig. 2). Both the polyclonal and the monoclonal antibodies recognized purified rGPA (Fig. 2, lanes 5 and 6, respectively). When incubated under identical conditions, neither antibody recognized rCNTF (lanes 11 and 12). When tested against a crude bacterial lysate (lanes 1 and 2) from a GPA overexpressing strain of E. coli, both the polyclonal and monoclonal antibodies recognized a 23 kDa band that comigrated with purified rGPA. The polyclonal sera also detected a higher molecular weight band (lane 1). Passing of the anti-GPA polyclonal sera over a GPA-affigel column abolished the specific immunoreactivity (lanes 4 and 8). The GPA antibodies did not cross-react with rCNTF (lanes 7 and 10); likewise, CNTF specific polyclonal antibodies were unable to recognize rGPA (lane 3). GPA antibodies were also able to deplete extracts of embryonic eye of a significant proportion of their ciliary neurotrophic activity (see below).

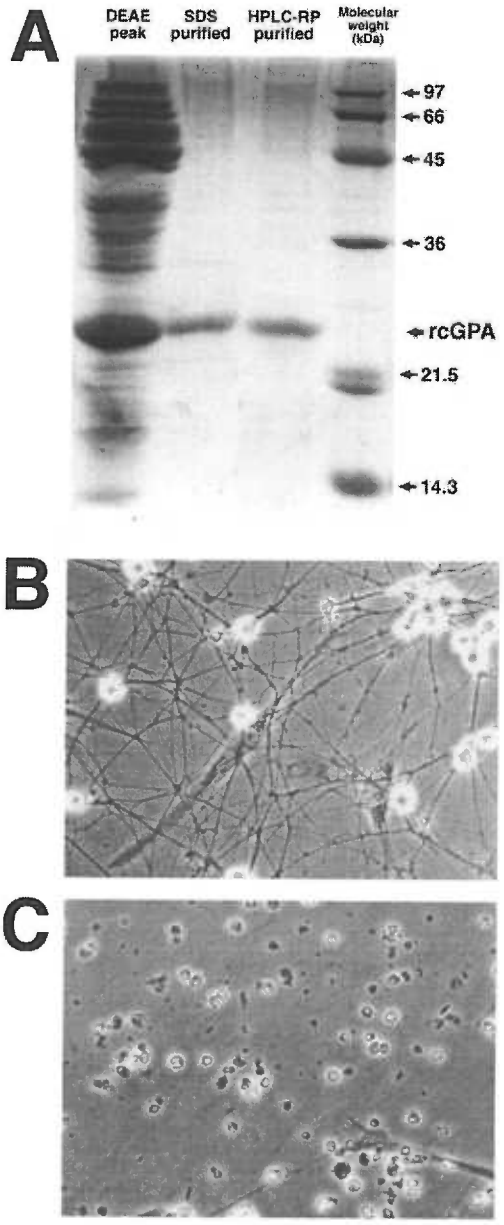

Figure 1. **Purity and biological activity of recombinant chicken GPA**. Panel *A*, aliquots of rGPA purified by protocols A and B were run on an SDS-PAGE gel and stained with coomasie dye to assess levels of contaminating proteins. Despite the more lengthy purification scheme of protocol B, both preparations were of about equal purity. SDS-purified GPA was subsequently used for antibody production and bioassays. Reverse phase purified GPA was used for monoclonal antibody production and for coupling to Affigel beads. Panels *B* and *C*, recombinant GPA supports survival of avian ciliary ganglion neurons in culture. E8 ciliary ganglion neurons were cultured in the presence (*B*) or absence (*C*) of 1 ng/ml rGPA for 3 days. Specific activity of rGPA varies between preparations, but is at least as potent as GPA purified from adult sciatic nerve.

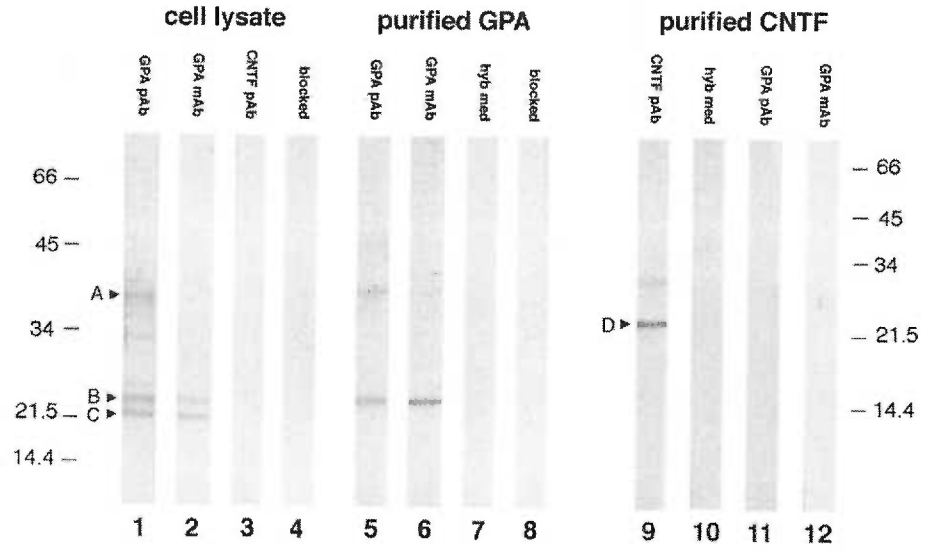

Figure 2. **Specificity of antibodies against GPA.** The specificity of polyclonal and monoclonal antibodies was monitored by western blot against a crude extract of a GPA over-expressing strain of E. coli (lanes 1-4). Recombinant GPA expressed under the conditions used here represents only a small percentage of total cellular protein and is not the most abundant protein. Cross reactivity to CNTF was determined by incubating GPA antibodies with equal amounts of purified rGPA (lanes 5 -8), and to purified rat CNTF (lanes 9-12). Both GPA polyclonal and monoclonal antibodies recognized a 23 Kda band in a crude bacterial lysate (lanes 1 and 2, respectively, marked B) and recognize the purified product (lanes 5 and 6). A 42 Kd band was often detected in both the crude lysate and in the purified preparations of rGPA (marked at A), which may represent a dimer. The band at C may represent a degradation product. Polyclonal anti-GPA sera passed over a GPA-affigel column abolished this immunoreactivity (lane 4), and unconditioned hybridoma medium had no specific signal compared to the monoclonal anti-GPA 13p (lane 8). Anti-CNTF antibodies were tested on rGPA and anti-GPA polyclonal sera on purified rat CNTF. Polyclonal anti-CNTF antibodies failed to recognize chicken GPA (lane 3) under identical conditions where an equal amount of rat CNTF (1 μg) can clearly be detected (lane 9) when tested under identical conditions (marked D).

Immunolocalization of GPA.

GPA in sciatic nerve. Consistent with the low abundance of GPA observed when it was purified from sciatic nerve[53], or from embryonic eye[54], GPA-like immunoreactivity (GPA-IR) proved challenging to detect. GPA immunoreactivity in tissues was best detected using a double peroxidase/ anti-peroxidase (PAP) staining protocol on floating sections of sciatic nerve or whole mounts of uveal layer (choroid, iris, and ciliary body, see Fig. 4). In rat sciatic nerve, cross sections stained with rabbit anti-rat CNTF showed dense staining around nerve axons, characteristic of myelin staining in Schwann cells [59, 60, 71, 82-84] (Fig. 3, Panel A and inset). Rat sciatic nerve sections incubated with normal rabbit serum showed no specific staining (Panel C). GPA polyclonal antibodies on chick sciatic nerve resulted in a staining pattern similar to that of CNTF-IR in rat sciatic nerve (compare Panels A and B). Preabsorbing the anti-GPA using a GPA-affinity column abolished the GPA-IR on chick sciatic nerve (Panel D). In longitudinal sections, GPA-like immunoreactivity (GPA-IR) was localized to the cytoplasm of Schwann cells and closely resembled CNTF staining in adult rat sciatic nerve (not shown). Whereas CNTF immunoreactivity could readily be detected in longitudinal sections, GPA-IR was extremely faint and could be found in only a few cells. To check for cross-reactivity of GPA and CNTF antibodies, GPA antibodies were used to stain rat sciatic nerve and CNTF antibodies chick sciatic nerve sections. Neither combination of antibody and tissue resulted in specific immunoreactivity.

GPA in ciliary muscle. The targets of the ciliary neurons in the ciliary ganglion are the muscle fibers of the ciliary body and iris (see Fig. 4a). The detection of GPA in whole mounts of ciliary body and iris was complicated by the thickness and density of the tissue and the high degree of pigmentation in the iris. GPA-IR, however, could be detected in those areas where pigmented iris cells were removed and the underlying ciliary processes were exposed (Fig. 5). GPA-IR appeared in the ciliary muscle as fairly dense staining in

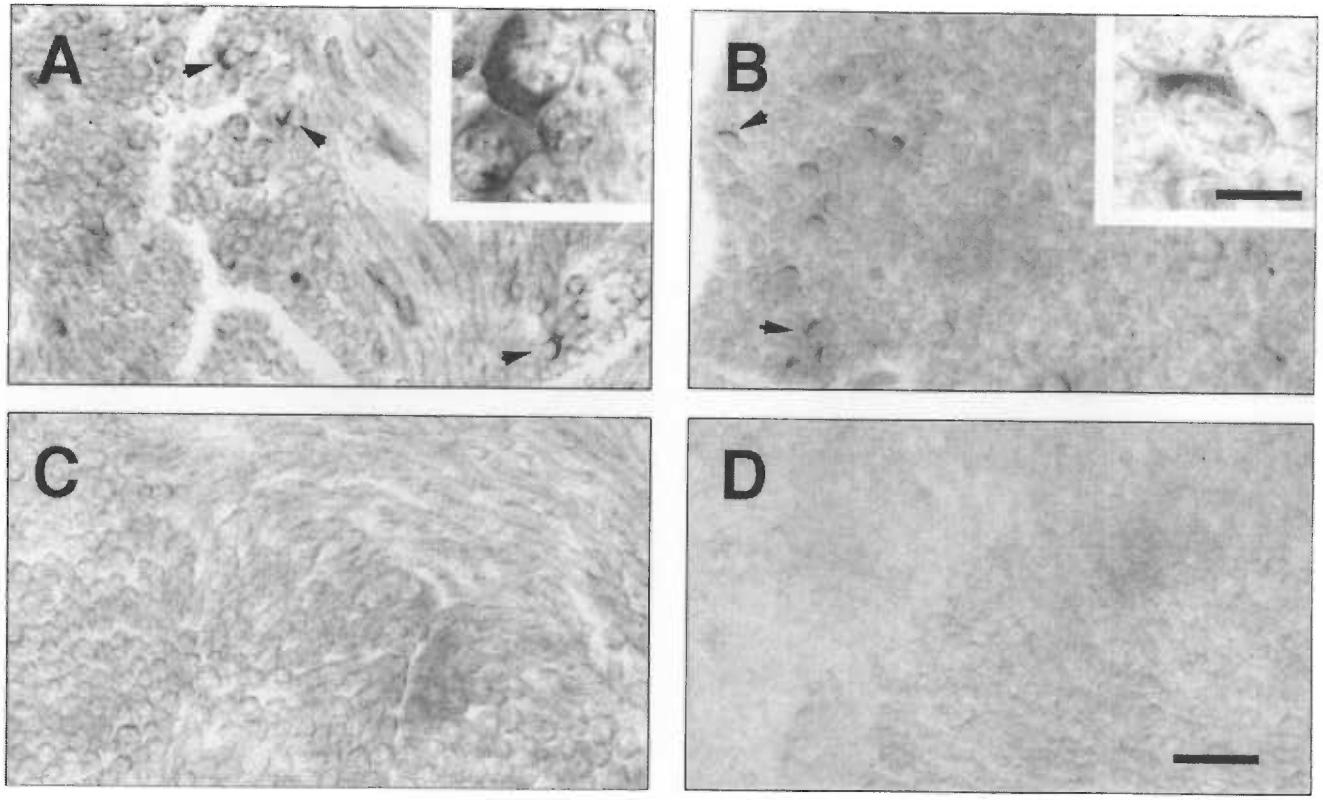

Figure 3. **GPA-IR in sciatic nerve.** The ability of polyclonal anti-GPA antibodies to recognize GPA in chick tissues was determined by performing immunocytochemistry on 20 μm adult chicken cryostat sections, stained floating. Sciatic nerve sections were stained with either rabbit anti-GPA sera (Panel *B* and inset), or with the same sera blocked by passing over a GPA-affigel column (*D*). For comparison 20 μm adult rat sciatic nerves were stained with rabbit anti-rat CNTF antisera (Panel *A* and inset) or NRS (*C*). GPA-like immunoreactivity strongly resembled CNTF expression, but was of much lower intensity and was present in only a small subset of cells. Bar = 20 μm, inset bar = 3.2 μm.

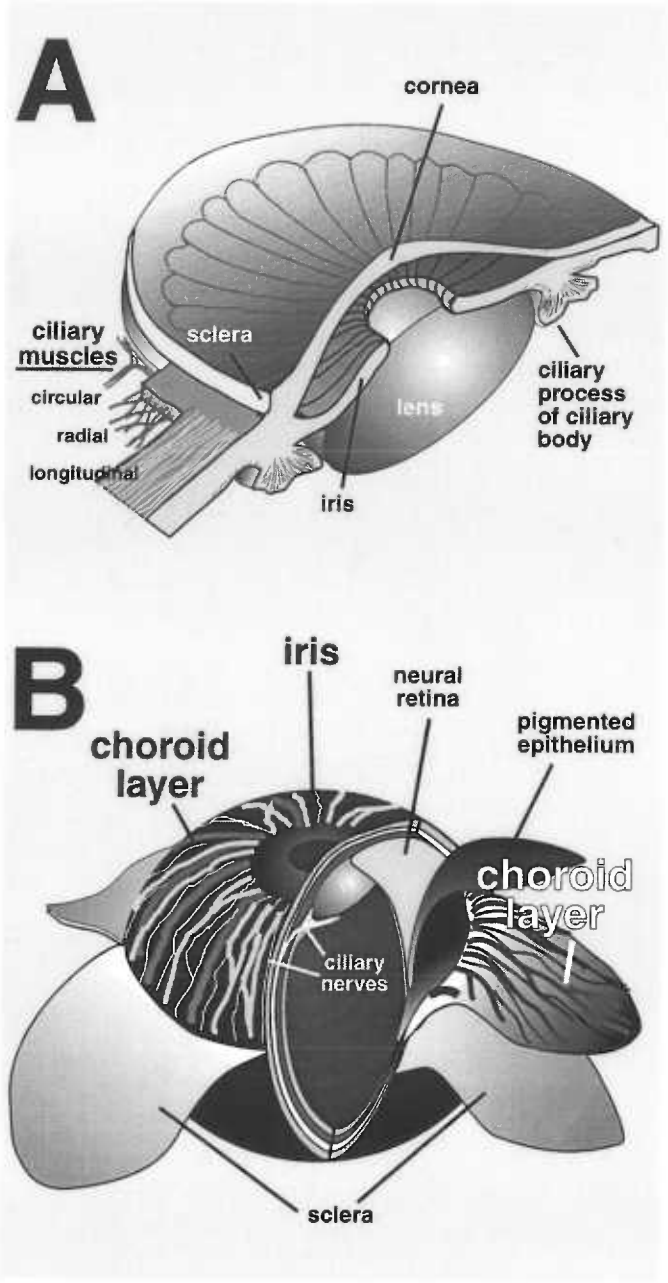

Figure 4. **Diagram showing the location of CG targets.** The ciliary ganglion lies at the back of the eye and contains two populations of neurons: choroid neurons that innervate smooth muscle cells surrounding choroid arteries in the choroid layer and ciliary neurons that innervate the ciliary body and iris. Panel *A*, cutaway view of the anterior portion of the eye showing the location of the ciliary neuron targets. Ciliary neurons innervate both the muscle fibers of the ciliary body and the sphincter muscle within the innermost portion of the iris. Panel *B*, the choroid layer is a highly vascularized layer that supplies blood to the other eye layers and can easily be isolated by gently peeling apart the three eye layers and removing the pigmented epithelium with a cotton swab.

individual muscle fibers (Fig. 5, arrowheads, Panel A). No such staining was seen when the GPA hybridoma conditioned medium was substituted with unconditioned medium (Fig. 5, Panel B). Overall, the staining was more intense at E12 for GPA-IR in the ciliary muscle than it was in any region of the choroid layer at the same age (see below).

GPA in the choroid layer of the eye. The targets of the choroid neurons of the ciliary ganglion are the smooth muscle cells of the arteries of the choroid layer. The choroid layer lies between the neural retina and the sclera of the eye (Fig. 4b), and is a complex tissue consisting of many small veins and arteries and extensive capillaries[85]. To determine if GPA is present in the smooth muscle cells surrounding choroid arteries, or in non-target cells located in the same tissue, we carefully removed the choroid layer from E12 chick eyes (Fig. 4b) and stained for GPA-IR using both polyclonal and monoclonal anti-GPA antibodies. Faint, but distinct GPA-IR was detected in the choroid layer at E12. Choroid layers stained for smooth muscle specific actin (SMSA) to identify the arterial vasculature had immunoreactivity that closely resembled the GPA-IR staining pattern (Fig. 6, compare Panels A and C). The amount of GPA-IR varied between different arteries, partially due to the fact that some arteries are surrounded by thicker layers of smooth muscle cells and some arteries are larger than others. Staining with polyclonal antibodies against GPA revealed the same pattern of immunoreactivity as that of the monoclonal antibodies, but the staining was less distinct (Panel E). At higher magnification, the GPA-IR localizes to cells with the elongated morphology characteristic of smooth muscle cells (arrowheads, panels B, D, and F). Controls including staining with unconditioned hybridoma medium or with GPA antiserum passed over a GPA-affinity column abolished this distinct pattern of staining (Panel G). Rat CNTF immunoreactivity was not detected in choroid (Panel H).

Although the similarity in the pattern of staining between anti-GPA and anti-SMSA suggested that GPA was being produced by smooth muscle cells surrounding arteries in the choroid layer, it was also possible that GPA-IR could be in endothelial cells of the arteries.

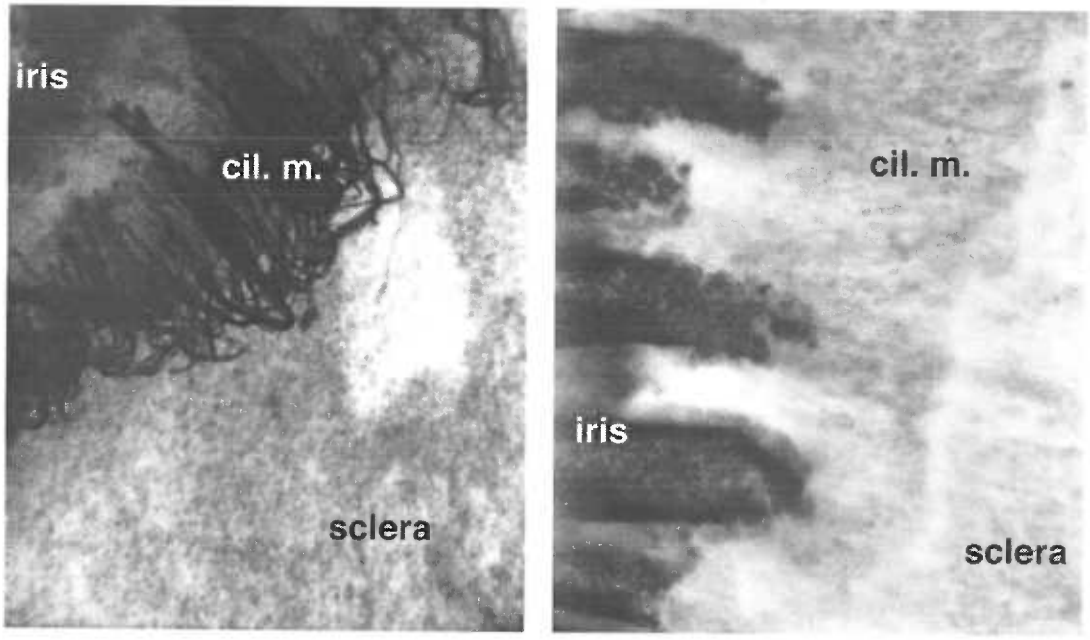

Figure 5. **GPA-IR in ciliary muscle.** E12 ciliary bodies and iris were stained with either anti-GPA 13p monoclonal antibody (*A*) or with unconditioned hybridoma medium (*B*). Panel *A*, GPA-IR localized to ciliary muscle fibers within the ciliary body. Dark areas in Panel *B* represent pigmented cells in the iris. Ciliary muscle, cil. m. Bar = 20 μm.

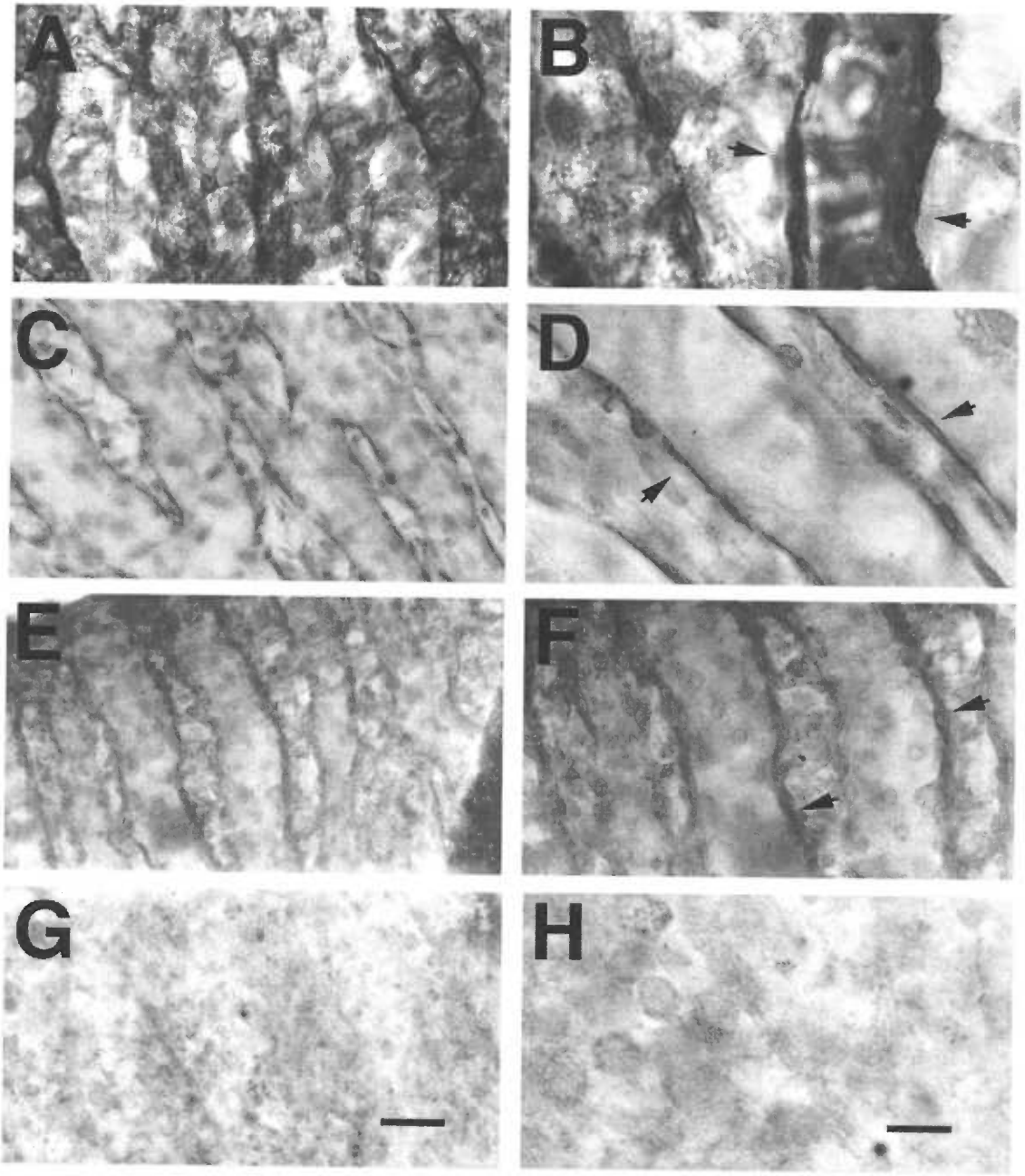

Figure 6. **GPA-IR in the choroid layer of the eye. Expression of GPA-IR in vascular smooth muscle cells of the choroid.** Intact choroid layers were dissected from E12 chick eyes, fixed, and stained for either smooth muscle specific actin (SMSA) (Panels *A* and *B*), GPA with anti-GPA polyclonal antibodies (Panels *C* and *D*) or with monoclonal anti-GPA13p (Panels *E* and *F*), with GPA polyclonal sera passed over a GPA-affigel column (*G*), or for CNTF with anti-CNTF polyclonal sera (*H*). All tissues were stained simultaneously using a double peroxidase-anti-peroxidase protocol with nickel enhancement. GPA like immunoreactivity (*C-F*) displayed the same staining pattern as that of SMSA (*A* and *B*). Arrowheads indicate cells with smooth muscle morphology that outline blood vessels. Panels *A, C, E,* and *G* are low magnification, bar = 20 μm. Panels *B, D, F,* and *H* are high magnification, bar = 8 μm.

Using double immunofluorescence we colocalized SMSA and GPA in choroid tissue (Fig. 7, arrowheads, Panels A and B, respectively). To exclude the possibility that the GPA antibodies cross-react with an epitope in smooth muscles, we stained chick amnion and blood vessels in the chorioallantoic membrane- rich sources of smooth muscle. Neither the polyclonal or monoclonal GPA antibodies were able to yield specific staining, even though both structures stained strongly for smooth muscle specific actin (data not shown).

Another possible source of GPA in eye could be myelinating Schwann cells of the nerves present within the choroid layer. We incubated E12 choroid layers with either anti-P_0 (a major myelin protein), anti-neurofilament, or with anti-GPA antibodies and compared the staining pattern for each. While anti-neurofilament antibodies stained a meshwork of large and small fibers throughout the choroid layer, P_0 staining was found in only a few large nerves coursing through the choroid layer. GPA-IR was not associated with either the P_0 or the neurofilament staining pattern (data not shown).

Comparison of GPA expression before, during, and after the cell death phase. Staining of the choroid layer at various developmental ages with polyclonal anti-GPA antibodies revealed that GPA-IR was undetectable until E10, where it faintly outlined blood vessels (Fig. 8, Panels A and B). GPA-IR arose strongly in expression by E15 (Panels C and D) through E17 (Panels E and F), where it appeared to level off with E19 showing only a slightly higher level of immunoreactivity (data not shown). Despite the variations in GPA-IR levels, the overall staining pattern in the vasscular bed changed little over time, with only the intensity of staining within smooth muscle cells being more intense.

As a more quantitative measurement of changes in GPA levels, whole extracts from eyes of various ages were assayed for neurotrophic activity. To ensure that the neurotrophic activity being measured reflected predominantly GPA and not other neurotrophic molecules, such as fibroblast growth factors [53], eye extracts were extensively

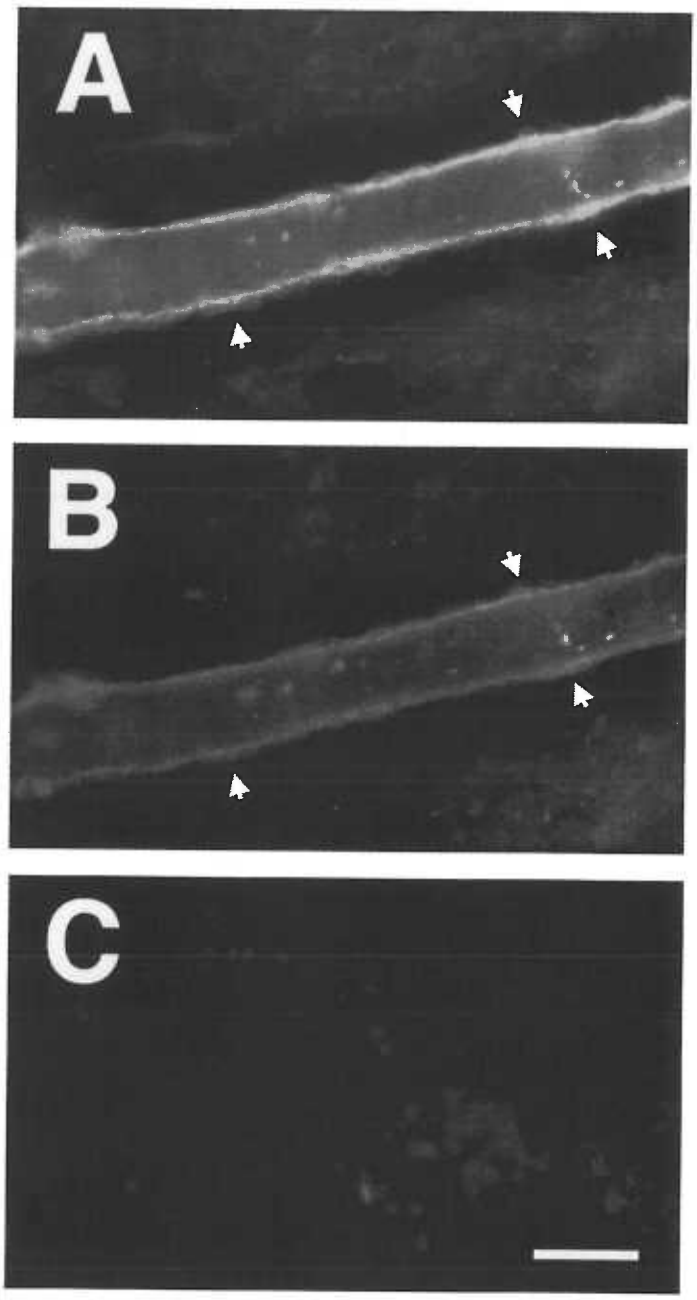

Figure 7. **Expression of GPA-IR in the vascular smooth muscle of the choroid layer of the eye**. E17 choroid layers were simultaneously stained for GPA with rabbit anti-GPA polyclonal sera and mouse anti-smooth muscle actin. Fluorescein labeled GPA (Panel *B*, arrows) colocalized to the same smooth muscle cells labeled by rhodamine coupled SMSA antibodies (Panel *A*). Another choroid layer stained for only SMSA using rhodamine labeled primary antibodies (Panel *C*) showed no significant fluorescence in the fluorescein channel. Arrows in *A* and *B* indicate cells lining blood vessels containing immunoreactivity. Note the coincidence in staining for SMSA and GPA. Scale bar = 20 μm.

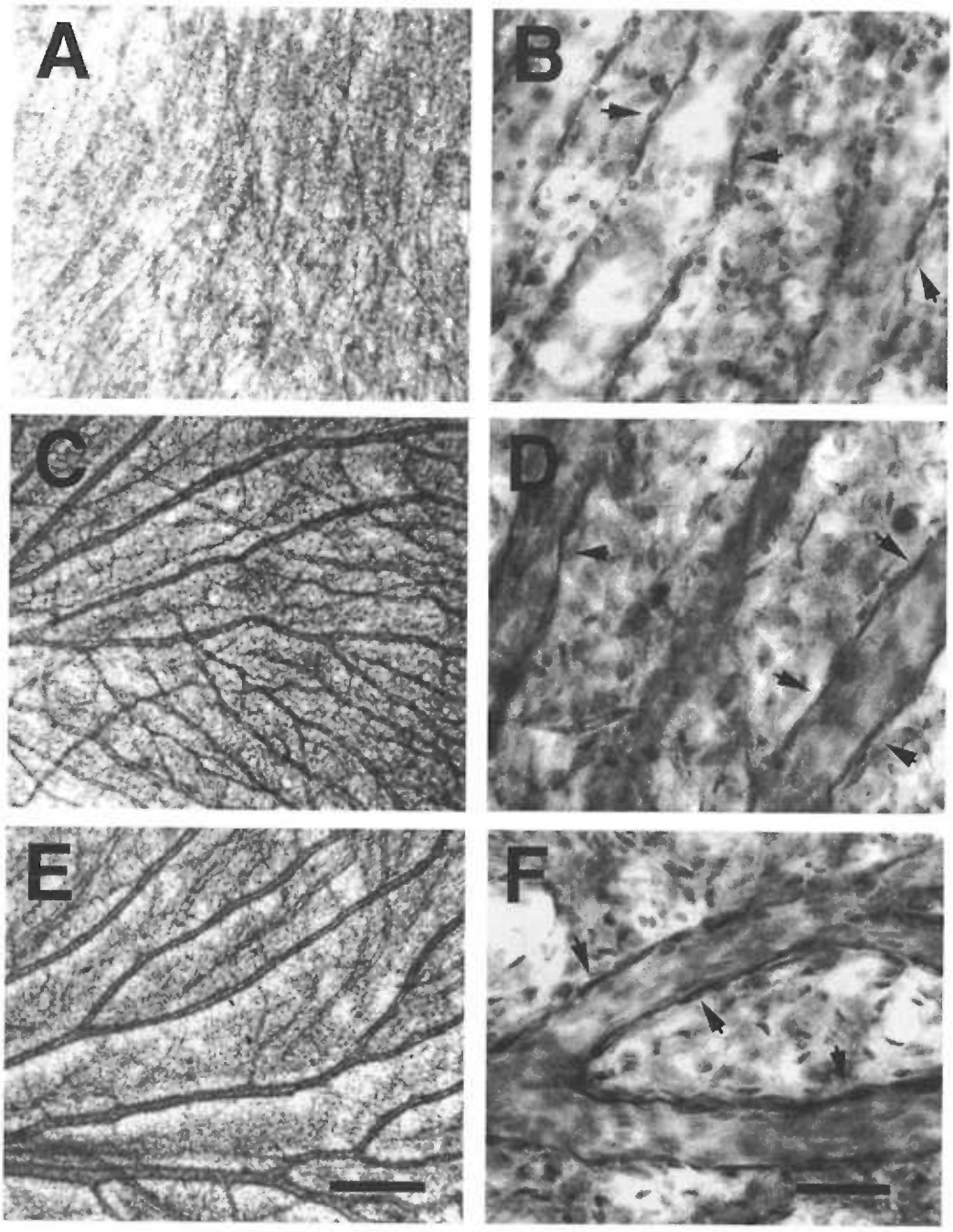

Figure 8. **GPA-IR in the choroid during CG neuron cell death.** Choroid layers were dissected from different age eyes, fixed, and stained for GPA using rabbit polyclonal sera. *A* and *B*, E10; *C* and *D*, E15; E and F, E17. Panels *A*, *C*, and *E* show at low magnification (bar = 20 μm) GPA staining of choroid layers at different ages. GPA-IR increases strongly at E15 and remained high thereafter. At higher magnification (Panels *B*, *D*, and *F*, bar = 8 μm) GPA staining intensity can be seen to increase per cell at later developmental ages and the number of cells stained is greater. Arrows in *B*, *D*, and *F* indicate smooth muscle cells of arteries containing immunoreactivity of GPA. Scale bars = 20 μm for *A, C, E,* 8 μm for *B, D,* and *F.*

dialyzed to remove small molecules, fractionated over a DEAE column, then passed over a heparin-agarose column to remove heparin binding growth factors. Comparing E9, E12, and E18 eye extracts prepared simultaneously and adjusted to equal volume, showed that E9 extracts already had significant GPA-like neurotrophic activity (Fig. 9b). E12 extracts were found to have about the same level of activity as E9, whereas E18 extracts had 12-fold more neurotrophic activity than E9. Of the total GPA-like activity present within an enriched E18 eye extract, 92% of the activity could be removed with anti-GPA sera (Fig. 9a). As a control, an identical aliquot was immunodepleted using NRS and only 11% of the activity could be removed. Boiling or protease treatment, on the other hand, depleted the neurotrophic activity to background levels (medium alone).

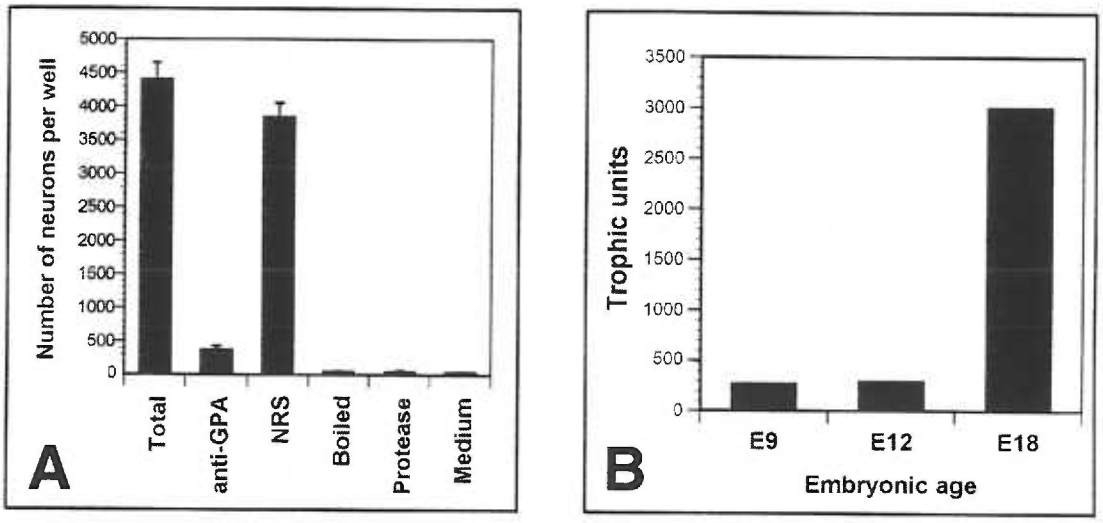

Figure 9. **Quantification of GPA in the eye during CG neuronal cell death.** Whole eye extracts were prepared from E9, E12, and E18 eyes and enriched for GPA by dialysis, and DEAE and heparin-agarose chromatography. Panel *A*, the amount of the remaining trophic activity that could be immunodepleted by anti-GPA polyclonal antisera was determined by incubating extracts with GPA antibodies pre-bound to protein A-agarose and testing the final sample on E8 neuronal survival assays. As a control an identical extract was treated with normal rabbit serum-protein A-agarose. Boiling the enriched extract for 30 minutes or incubation with proteinase K (1 mg/ml) for 20 minutes at 37°C could also remove the activity. Culture medium alone was used as a background measurement of activity. This is a representative experiment with each time point done in triplicate and the error bars indicates the SEM. Panel *B*, representative experiment showing the specific activity of different age eye extracts. Dose response curves were generated for each of the three time points in a short term survival assay (1 week). Neurons were plated in culture medium supplemented with either a saturating amount of GPA, medium alone, or with various dilutions of extract. All extracts were dialysed, DEAE fractionated, passed over a heparin agarose column and filter sterilized before use. Trophic units were calculated as the greatest dilution of extract that gave half-maximal results times the volume of extract. All extracts were adjusted to equal volume before testing.

Discussion

In order for a molecule to be a true target-derived trophic factor for CG neurons the following criteria must be met: 1) the molecule must be synthesized and released by the cells innervated by the neurons, 2) it must be present during part or all of the cell death phase; 3) addition of exogenous factor should rescue neurons from cell death; and 4) blocking the effect of the molecule should exacerbate cell death. We have previously purified and cloned GPA, a molecule that is similar in size and structure to rat CNTF, but is secreted from transfected cells[54]. We have now prepared a highly active form of recombinant GPA, isolated polyclonal and monoclonal antibodies against GPA, quantitatively depleted the ciliary neurotrophic activity from embryonic eye extracts by anti-GPA, and localized GPA-like immunoreactivity to cells innervated by ciliary ganglion neurons. These results establish that GPA meets the first two criteria described above for a target-derived trophic factor for CG neurons. Thus, the stage is now set for testing the effects of manipulating GPA levels *in vivo*.

Recombinantly expressed GPA was equal to or more active than native GPA, with an ED_{50} of about 1 pg/ml or about 0.05 pM. The difference in biological activity seen between native and recombinant GPA is probably a reflection of the different purification protocols used and the fact that detergents increase the specific activity and stability of rGPA preparations. Recombinant GPA also had a higher specific activity than that reported for recombinant forms of CNTF[86-89], perhaps reflecting the greater efficacy of a nonspecific molecule.

Since GPA was originally purified from sciatic nerve, we first tested our immunocytochemical staining protocol on sections of adult chicken sciatic nerve and compared the GPA staining pattern to that of anti-rat CNTF on sections of rat sciatic nerve. The distribution of GPA-IR within sciatic nerve closely resembled that of CNTF-IR. However, GPA-IR, was present in far fewer Schwann cells and within a given cell was of

much lower intensity compared to CNTF in rat tissue. The relatively lower amounts of GPA observed in chick versus CNTF in rat sciatic nerves was consistent with the approximately 10-fold higher purification required to isolate GPA (>80,000-fold) versus CNTF (10,000-fold). In addition CNTF antibodies were unable to immunolocalize GPA in chick sciatic nerve. Similarly, anti-GPA was unable to detect CNTF in rat sciatic nerve, indicating that although GPA and CNTF are structurally similar, they differ in antigenicity.

GPA immunoreactivity in the developing eye was localized to both types of target cells innervated by CG neurons. Staining of smooth muscle cells in the arterial vasculature was determined by double immunofluorescence using mouse anti-smooth muscle specific actin and rabbit anti-GPA. Due to interference from pigmentation, we were unable to observe immunolocalization of GPA in the iris; however, staining of fibers in the ciliary muscle was distinct and stronger in intensity than that within the choroid layer. The distribution of GPA immunoreactivity was in agreement with results obtained previously using bioassays of crude extracts from microdissected portions of the eye[47, 90, 91]. Our results also suggest that the trophic activity observed in explanted cultures of chick choroid[92] is likely to be GPA. Amnion, on the other hand, is highly enriched in smooth muscle and reported to possess a trophic activity for CG neurons[92], and did not have detectable levels of GPA immunoreactivity. Thus, the trophic activity in amnion may have been due to another molecule.

GPA immunoreactivity in the choroid appeared to remain low before and during CG cell death with a dramatic increase once cell death was complete. This was confirmed by bioassays of enriched supernatant of eye extracts under conditions where virtually all of the biological activity could be immunodepleted by antibodies against GPA. These results are in agreement with previous studies measuring CG trophic activity in conditioned medium from cultured CG targets removed from various stages of development[91]. In these previous studies the identity of the molecule responsible for the trophic activity was not known. The changes in GPA protein levels during development were also in agreement

with our previous observation that GPA mRNA levels in the developing eye[54], are low prior to cell death but increase significantly halfway through cell death.

It has been suggested that the high level of ciliary neuronal survival activity in crude eye extract is difficult to reconcile if survival activity is available only in limiting amounts[93]. However, it may be difficult to extrapolate from levels accumulated by cells to amounts that are actually released and made available to neurons. For example, we have previously shown that the release of GPA from transfected cells appears to be inefficient[54]. We have also observed that crude eye extracts contain multiple sources of trophic activity for CG neurons, that may not all be available to the neurons *in vivo*. In addition to a non-heparin binding macromolecular fraction that could be immunodepleted with antibodies against GPA, we also found dialyzable and heparin binding fractions with activity. The heparin binding fraction is likely to be acidic or basic FGF, neither of which are known to be secreted from cells.

The structural similarity of GPA to CNTF, together with our observations that GPA is expressed in Schwann cells of the sciatic nerve as well as target cells innervated by CG neurons, suggest that GPA is chicken CNTF. Interestingly, chicken CNTF differs from mammalian CNTF in that it is released by transfected cells and is expressed embryonically. Ironically, the expression of CNTF in the mammalian iris and ciliary body has never been examined. In fact, no reports have yet appeared as to whether CNTF knockout mice retain a pupillary reflex, a functional indicator of the presence of ciliary neurons, although it has been reported that CNTF is unable to rescue CG neurons from cell death in chicken embryos [4]. However, these studies used recombinant human CNTF which had an ED_{50} of 470 pg/ml. This is considerably lower than the specific activity of freshly purified recombinant chicken CNTF (1 pg/ml), and these experiments should be repeated with the homologous molecule.

Our results provide further support for the hypothesis that GPA, or chicken CNTF, is the target-derived neurotrophic factor that regulates CG neuron cell death *in vivo*.

Definitive proof of this hypothesis awaits *in vivo* experiments manipulating the availability of endogenous trophic activity in CG neuron targets. If the CNTF family members are found, it will be interesting to see if chickens have this factor as well or if CNTF in chickens serves a dual role that in mammals is divided between two molecules.

Acknowledgments

We would like to thank Genentech, Inc. for providing the pTrp expression vector and for their assistance in preparing rabbit anti-GPA sera and reverse phase purified GPA. We would also like to thank all of those who helped with the preparation of this manuscript, especially C. Gary Reiness. This project was funded by NINDS grant # NS25767.

Supplementary data to Chapter 1

A. Expression and purification of chCNTF in a polyhistidine-tag vector.

Rationale. The extremely low abundance of chCNTF in the chick embryo makes the availability of a recombinant form a necessity. Only 1.5 μg of chCNTF was obtained from 500 adult chick sciatic nerves (requiring a 80,000-fold purification)[53] and calculations based on purifications from E15 chick eye suggest that less than 1 ng is present per eye. The two purification protocols described in this chapter requires two weeks to complete from the start of the purification. Furthermore, the selection of the pTrp vector was based on the assumption that lower levels of expression in *E. coli* would avoid the problem of inclusion body formation often accompanied in bacterial expression of proteins. Rat, and especially chick CNTF, however, are much more hydrophobic than predicted from their primary sequence and expression in *E. coli* always resulted in the formation of large protein aggregates that were very difficult to dissociate. This reason, coupled with the need to produce purified chCNTF preparations more quickly led to the use of a second expression system and a third purification protocol. In addition, three tyrosines were genetically added to the carboxy terminus to aid in iodination of chCNTF needed for receptor binding studies [94-96]. Previous attempts at iodinating rat and chick CNTF resulted in too low of specific activity to be useful.

Methods. The coding region for chCNTF was inserted downstream of the PQE-8 expression vector (Qiagen) start site by subcloning into a HindIII restriction site. A HindIII site was added to chCNTF at the appropriate location by PCR. The vector was transformed into *E. coli jm101* and colonies were screened by PCR. Fourty-five colonies were screened, of which 32 contained the chCNTF insert. Five of the colonies were

selected and tested for induction of rchCNTF in the presence of IPTG. Eight liter cultures were grown up at 37°C, and induced at an optical density of 0.5 with 1 mM IPTG. Cultures were further incubated for 4 hours and harvested by centrifugation and extracts prepared as described for the two other protocols. ChCNTF was extracted by lysing bacterial cell paste in a lysozyme containing buffer supplemented with a cocktail of protease inhibitors and incubating at 37°C for 20 minutes. The cell extract was combined with 5 volumes of 6M guanidine-HCl, 0.1 M Na_2PO4, 10 mM Tris pH 8.0 and rocked gently for 30 minutes at room temperature. The lysate was centrifuged at 41,000 x g for 15 minutes at 4°C, then passed over a Nickle-agarose column (Qiagen) and washed with 10 volumes of column buffer (8M urea, 0.1 M Na_2PO_4, 10 mM Tris, pH 8.0, 0.05% Triton-X100), followed by 5 volumes each of column buffer pH 6.3 and 5.9. ChCNTF was eluted in 10 volumes of column buffer pH 4.5, the protein containing fractions determined by the method of Bradford [97] pooled, then reloaded onto the nickel column equilibrated with column buffer, pH 8.0 to remove the urea. The column was washed with 20 volumes 50 mM NaMOPS pH 7.2 (Sigma, St. Louis, MO), and chCNTF eluted with 20 mM NaMOPS, 0.1% SDS, pH 7.2. SDS was partially removed from the preparation by desalting over a Sephadex G10 column (Sigma), concentrated by ultrafiltration, then stored at -80°C in 20 mM NaMOPS, 50% glycerol, 0.5% CHAPS. The final purity of the product was about 95% of total protein as determined by densitometry of all protein bands on a coomassie stained SDS-PAGE gel of purified chCNTF (Fig. 1).

Results and conclusions. No direct comparison of biological activity was made between rchCNTF produced by the three different purification protocols. Such a comparison would only be relevant if the preparations were made within a few weeks of one another, as chCNTF, like other neurotrophic factors, loses significant biological activity even when stored at -80°C. After one year of storage at -80°C in the presence of 0.5% beta-octyl

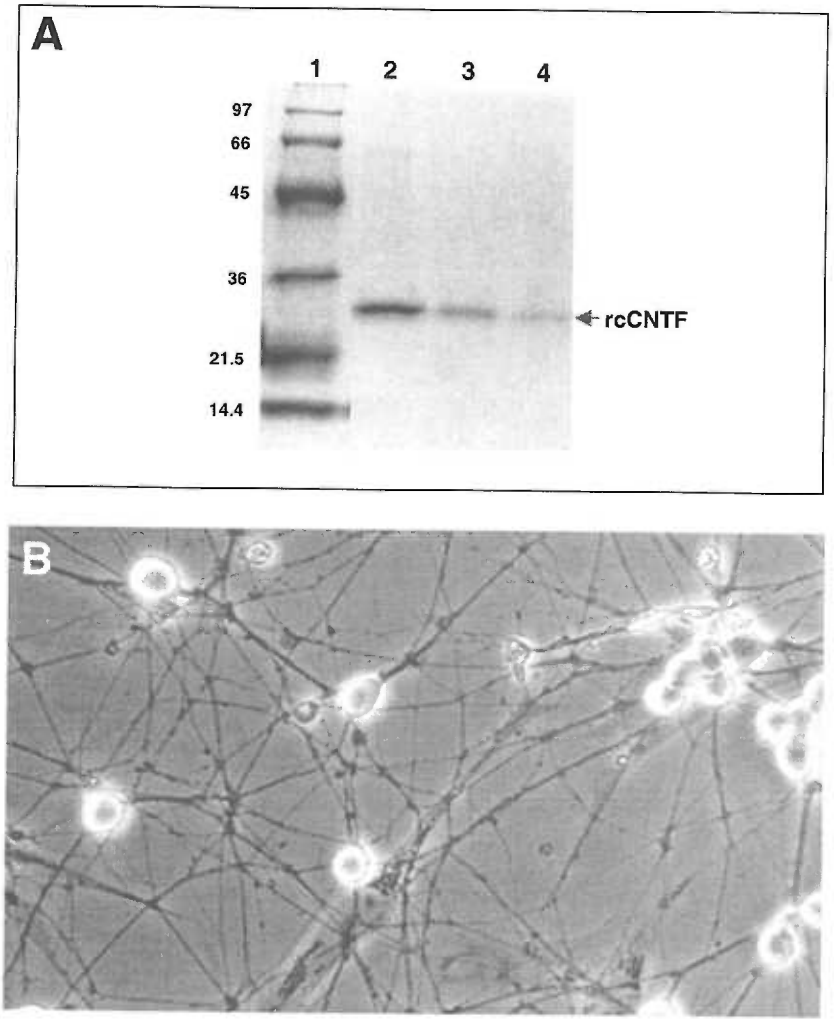

Figure 1. **Purification of chick CNTF by nickel column chromatography.** Chick CNTF expressed with a polyhistidine tag could be resolved in one chromatographic step, Panel *A*. various amount of the purified factor were loaded loaded in lanes 2-4. As predicted, polyhisitdine tagged chCNTF migrates as a larger protein compared with chCNTF. All purified preparations of chick CNTF were able to support CG neurons in long term cultures, Panel *B*.

glucoside and glycerol, chCNTF produced by any of the three methods lost about 10-fold activity, and after three years at least 50-fold activity was lost. Storage at -20°C in glycerol appears to retain more biological activity in long term storage. Addition of either beta-octyl glucoside, CHAPS, Triton-X100, or small amounts of SDS increase biological activity by as much as 100-fold. A comparison of individual bioassays on preparations made by the three different protocols is given in Table 1.

Table 1. Typical properties of purified material from the two expression systems and three purification protocols.

preparation	yield per preparation (mg)[1]	purity (percent)	activity (ED_{50})
pTrp-chCNTF, SDS-purified	5	97	1 pg/ml
pTrp-chCNTF, reverse-phase purified	2	98	20 pg/ml
pHis-chCNTF, Nickel purified	15	95	30 pg/ml

[1]. Values given are representations of typical values, actual values varied between preparations.

ChCNTF expressed and purified by this protocol proved to be the most effective means of obtaining milligram quantities of the factor. Instead of a two week protocol, chCNTF could be prepared in 2 days with much higher yield and similar activity.

Subsequent chCNTF preparations were critical for characterizing biological activity and high affinity binding sites on sympathetic neurons[94] for characterizing the chCNTF receptor GPARα[95] and for characterizing high and low affinity binding sites on CG neurons [96].

B. Distribution of chCNTF in the chick embryo analyzed by Northern blot analysis.

Rationale. Most neurotrophic factors that have been tested are known to affect more than one population of neurons and are found in numerous tissues. For example, CNTF can support survival of spinal cord motor neurons[98-102], rat cortical-spinal neurons[103], thalamic nuclei neurons[104], hippocampal neurons[111], Purkinje cells[105], facial motor neurons[106], retinal ganglion neurons[107-109], nodose ganglion cells[110], acoustic ganglion neurons[113, 114]sensory neurons[48, 53, 112, and sympathetic neurons[48, 53], in addition to CG neurons[48, 53]. CNTF is also distributed in locations outside the targets of neurons and may serve a role as a lesion factor[69, 83, 115, 116]. Rat CNTF expression can first be detected at P7 in sciatic nerve and its expression goes up substantially thereafter and is maintained at relatively high levels in the adult[51, 70]. It has also been detected in adult optic nerve and the olfactory bulb, but not in skeletal muscle, liver, spleen, or lung of adult rats[70]. Rat CNTFRα, however, is expressed during development and is found in many areas of the brain, spinal cord, muscle, and fat cells[117, 73]. This led us to question whether chCNTF might be localized in other areas of the embryo.

Methods. Tissue collection and preparation of RNA samples. Tissues were collected from the appropriate age white leghorn chick embryos incubated for the appropriate number of days and staged according to Hamburger and Hamilton[118]. All tissues were

immediately immersed in liquid nitrogen and stored at -80°C until processed for RNA. RNA was extracted from tissue samples by directly thawing into Tri-reagent (Molecular Research Center, Cincinnati, Ohio) and processed according to the manufacturer's directions. RNA pellets were resuspended in formazole, the purity and concentration determined by measuring the absorbency values at 260 and 280 nm, then stored at -80°C until ready for use. For Northern blots, 30 μg of total RNA was loaded per lane and run on a 1% agarose-6.6% formaldehyde gel. The RNA was transferred to nitrocellulose and prehybridized for 2 hours at room temperature, then hybridized overnight at 73°C in 50% formamide and 0.75M NaCl with a 123 bp chCNTF riboprobe adjusted to 1 $x10^6$ cpm/ml. A second probe for chick ribosomal protein subunit (chRPS) was added at $2x10^5$ cp/ml to the same hybridization solution. The blot was then washed in 0.05X SSC-1% SDS four times, dried, and exposed to a Molecular Dynamics phosphoimager screen for three days. Blots were quantified by taking the ratio of the chCNTF signal to chRPS signal in each lane.

Results and conclusions. RNA was isolated from neural retina and whole brain since these are areas that have been identified to express chCNTFRα. Leg and pectoral muscle were also chosen since CNTF can promote survival of motor neurons *in vitro* and *in vivo* and these are the targets of lateral spinal column motor neurons. Although previous studies failed to show the presence of chCNTF in E12 neural retina[54], an updated protocol using a shorter probe revealed the presence of the message in E9 through E19 neural retina (not shown). ChCNTF message was also detected in E19 brain, including cerebellum, but was not found in either pectoral or leg muscle (Fig 2). The levels in brain were slightly higher than that of E19 whole eye, which would include chCNTF the neural retina signal as well as that of the two targets. Expression of chCNTF in neural retina and brain suggests a possible involvement in the regulation of cell death or differentiation of other populations of neurons. Studies are underway to localize the source of expression by

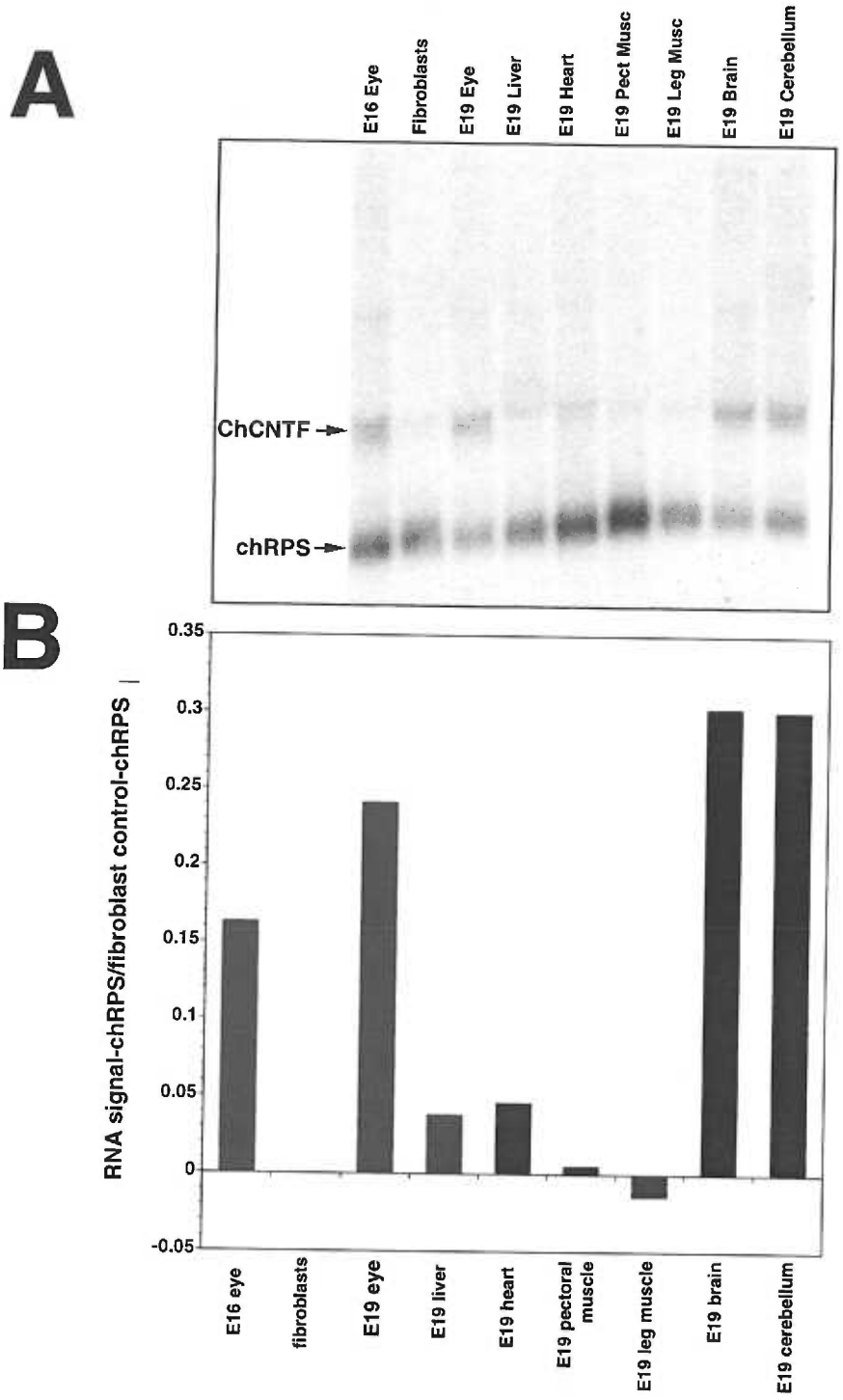

Figure 2. **Expression pattern of chCNTF mRNA in E19 embryos.** *A*, Chick CNTF message is readily detectable in brain, including the cerebellum, using a riboprobe specific for chCNTF. Approximately 30 μg of total RNA was loaded in each lane. B, The chCNTF signal in each lane was subtracted from a fibroblast negative control and normalized to a constuatively expressed gene, chRPS. The relative levels of chCNTF in each tissue is plotted.

chCNTF in muscle would argue against it playing a role in spinal cord motor neurons development. However, the critical phase of development when motor neurons become target-dependent and begin cell death occurs much earlier at E6[4]. It would have also been interesting to examine expression in the CG itself. However, since staining of chCNTF has proven to be ineffective on sections mounted to slides and Northern blot analysis too insensitive for such a small tissue, these questions will have to await analysis by PCR or RNAse protection methods.

C. Developmental expression of smooth muscle actin in the choroid layer of the eye.

Rationale. The reason cell death in the nervous system is thought to occur is to match the neuronal populations with the target fields they innervate. This would leave one to believe that the target has stopped developing and that the "match" that is created would be fine-tuned. But target tissues continue to develop as do the neurons and the rest of the embryo. During the first 6 days of chick development, the embryo roughly doubles in size each day. In the case of CG targets, the iris and choroid layers go through massive changes between E8 and E14. In the choroid layer, this is especially apparent since the layer is transparent and its morphology can be easily observed under bright-field microscopy. At E7, the choroid layer is very thin and is difficult to remove, but even at this age, small blood vessels can be readily seen. By E14, the tissues is much larger and thicker, and the blood vessels and capillaries are more numerous. By E17, blood vessels can easily be seen with the naked eye. For this study, choroid layers were collected at three ages and stained for smooth muscle actin as a measure of smooth muscle cell abundance on choroid arteries during and after the cell death phase.

Methods. Choroid layers were dissected from embryonic chick eye, fixed, and stained for smooth muscle alpha-actin as outlined in Chapter 1. The choroid layer is much smaller,

thinner, and more fragile at the younger ages of development. At ages younger than E9 it is difficult to isolate a large portion of the layer intact. Choroid layers were left attached to the sclera during staining to help prevent folding of the tissue. Smooth muscle actin staining was detected using a double-peroxidase-antiperoxidase staining protocol.

Results and conclusions. At E10, SMSA-IR was just detectable (Fig. 3). At high magnifications it could be seen on long, spindle-shaped cells dispersed sparsely around blood vessels. The presence of so few smooth muscle cells was surprising considering the CG is nearly one third the way through cell death. At E12, SMSA-IR is much more pronounced. The staining pattern parallels that of chick CNTF closely, with the exception that smooth muscle actin immunoreactivity is much more intense than chCNTF immunoreactivity. As far as could be determined, every artery seemed to contain smooth muscle cells and at higher magnification each artery appeared to have the majority of its walls covered by smooth muscle. By E17, the staining was very intense and blood vessels were very clearly outlined. In mature arteries, more than one layer of smooth muscle cells can surround an artery and at E17, some blood vessels appeared to have staining in cells more than one layer thick. The fact that levels of smooth muscle actin expression parallel the staining and the relative number of trophic units of chCNTF may partially explain why chCNTF levels increase so dramatically after the cell death phase (Chapter 1, Fig. 8 and 9). Since only a single marker was used to localize smooth muscle cells, it is not possible to discern how much of the increase in smooth muscle actin expression was due to an increase in the number or size of smooth muscle cells versus the amount of actin expressed by each cell, except at the level of comparing the morphology of the arteries at each age and the relative staining intensity. Nevertheless, the amount of smooth muscle obviously expands greatly during and after the cell death phase is taking place.

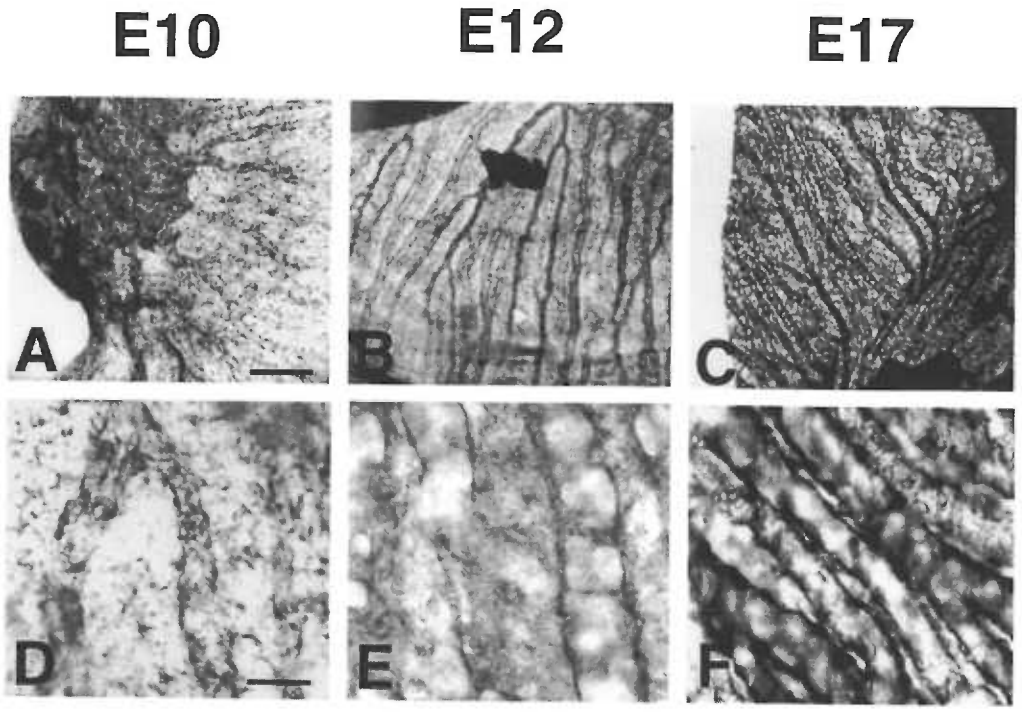

Figure 3. **Developmental expression of smooth muscle actin in the choroid layer of chick eye.** Choroid layers were dissected from different age eyes, the pigmented epithelium removed, fixed, and stained for smooth muscle actin usuing a specific monoclonal antibody. Smooth muscle actin expression is weak, but detectable at E10 (*A* and *D*), increases significantly by E12 (*B* and *E*), then dramatically by E17 (*C* and *F*). calibration bars = 20μm for A-C, 8 μm for D-F.

Chapter 2

Elevation of chick ciliary neurotrophic factor levels *in vivo* enhances CG neuronal survival.

Thomas P. Finn and Rae Nishi

Abstract

Chicken ciliary ganglion (CG) neurons undergo target-dependent cell death during embryonic development. We have hypothesized that the number of CG neurons surviving cell death is controlled by regulating the availability of a target-derived molecule, chicken ciliary neurotrophic factor (chCNTF), also known as growth promoting activity (GPA). In order to test this hypothesis we have overexpressed chCNTF in embryos using a replication competent retroviral vector, RCASBP(A). Using an *in vitro* survival assay on CG neurons we confirmed that an average 2.8-fold increase in CNTF-like biological activity was observed in eyes from embryos infected with RCAS(BP)A-chCNTF. Staining for viral gag protein and chCNTF expression revealed that the virus had spread extensively and evenly throughout the eye, and that chCNTF immunoreactivity was significantly higher in RCASBP(A)-chCNTF infected embryos than in uninfected embryos or embryos infected with viral vector alone. No gross morphological changes in the size or shape of the eye were observed and the iris and choroid layer, which are the targets of CG neurons, were normal. RCASBP(A)-chCNTF embryos has an average of 23% more neurons than control embryos at embryonic day 14, and in some individual cases as many as 72% of the neurons were rescued from cell death. Counts of CG neurons prior to cell death (E8) in control and CNTF overexpressing embryos showed no difference in neuronal number, indicating that CNTF did not alter the number of neurons generated by proliferation and differentiation of neuronal precursors. Taken together with previous studies showing expression of chicken CNTF (GPA) at the correct time and location in CG targets, these results support the role of chCNTF as a target-derived neurotrophic factor.

Introduction

A popular model for how neuronal cell death is regulated in the developing nervous system is that neurons compete for limiting amounts of neurotrophic factors produced by the targets they innervate. Neurons become dependent upon their targets for survival shortly after they have innervated them. A number of neurotrophic factors have either been purified from or found expressed in particular targets. Understanding how the limited availability of these factors regulates neuronal cell death *in vivo* is an area of intense research.

The avian ciliary ganglion is an attractive system for the study of target-supplied neurotrophic factors because it is a relatively simple system. Neurons of the avian ciliary ganglion (CG), like most neuronal populations, undergo a specific period of cell death during development. Since the ganglion only contains two populations of neurons, each of which innervates specific, well-defined targets in the eye[35], the CG is very amenable to the study of neuron-target interactions. In particular, this system is well suited for examining trophic dependency and how true target-derived neurotrophic factors function and are regulated. Choroid neurons innervate smooth muscle cells surrounding arteries within the choroid layer and CG neurons innervate the sphincter and ciliary muscle of the iris and ciliary body, respectively. Choroid and ciliary neurons become dependent upon their target beginning at about E8[37], and the number of neurons are reduced from approximately 6500 neurons to 3200 by E14. Removal of the eye at a time before CG cell death begins ultimately results in the death of the majority of CG neurons[38] while addition of extra target tissue enhances cell survival[11]. CG neurons become post-mitotic after E6[37] and, therefore, the level remaining at E14 is a reflection of those neurons which have successfully competed for target-supplied factors.

The identity of the CG target-derived factor(s) still remains to be established. Candidate molecules include acidic and basic fibroblast growth factor (aFGF, bFGF)[53],

glial-derived neurotrophic factor (GDNF)[119], and chick CNTF, also known as GPA[53, 54]. The bulk of evidence supports chick CNTF (chCNTF) as the most likely candidate for this role. ChCNTF message and protein are present in the target tissue at the time the neurons are dependent upon their targets for survival [54, 120], and high affinity receptors for chCNTF are present in the ganglion[95, 96]. ChCNTF is a potent neurotrophic factor *in vitro* with an ED_{50} of about 10-30 pg/ml and can support nearly 100% of the neurons in culture [53]. While rat, rabbit, mouse, and human CNTF are generally considered to be non-secreted or very poorly secreted[51, 52], chCNTF can readily be detected in choroid smooth muscle conditioned medium or in cell lines transfected with chCNTF[54]. The secretion of chCNTF appears to be due to the presence of an internal signal sequence between amino acid residues 40 and 60[121]. Together, this is strong circumstantial evidence for an involvement of chCNTF in the regulation of CG cell death. Definitive evidence, however, will require altering chCNTF levels *in vivo* and assessing the effect it has upon CG neuronal cell number.

An important criterion for demonstrating that a neurotrophic factor is limiting *in vivo*, and therefore responsible for inducing cell death, is to determine whether making more factor available rescues neurons from cell death. The most direct means of elevating the levels of a neurotrophic factor *in vivo* would be to inject pure factor directly into the embryo during cell death. However, if injection fails to rescue neurons, then it is not clear whether sufficient quantities of active factor were made available to the developing neurons. An alternate means of delivering a more consistent supply of factor directly to neuronal terminals would be to express the cDNA encoding the factor in neuronal target tissues. A vector well suited for such a study is the retroviral vector, RCASBP(A).

RCASBP(A) is a replication competent retroviral vector derived from Rous sarcoma which has had the src gene deleted. The trans gene of interest is inserted in place of src and the genome encodes functional gag, pol, and env genes. As the virus spreads, the transgene is inserted into the genome of infected cells and expressed using the viral LTR,

and widespread expression of the transgene is rapidly achieved. In the present study, we inserted the coding region of chCNTF into RCASBP(A) and harvested infective viral particles after transfecting chicken fibroblasts *in vitro*. Chicken embryos were infected at early stages of development with viruses containing chCNTF or viral vector alone. Ciliary ganglia were harvested after cell death and the impact of chCNTF overexpression on neuronal survival was analyzed.

Materials and Methods

ChCNTF bioassays. Short term (2-5 days) CG neuronal survival assays were performed according to[53], using E8 (Stage 34) ciliary ganglia, except that ganglia were plated at a density of 0.75 ganglia per well in 48-well plates. Neuronal survival was determined by counting the number of large, phase-bright cells with processes greater than 3 cell diameters in 4 day cultures. Sixteen fields of view were counted per well and all samples were tested in duplicate or triplicate.

Preparation of extracts. Fibroblast or choroid smooth muscles cultures were collected from two 100-mm plates, pelleted by centrifugation, then resuspended in a small volume of 10 mM NaMOPS, 1 mM EDTA pH 7.4 with a cocktail of protease inhibitors. Extracts were prepared by sonication for 30 seconds on ice with a probe type sonicator and the lysate centrifuged at 30,000 x g for 20 minutes at 4°C. Eye extracts were prepared by a similar protocol except samples were adjusted to 0.5 ml buffer for each eye used and homogenation with a Tekmar tissue homogenizer for 45 seconds on ice prior to sonication. All lysate supernatants were concentrated by ultrafiltration 10-fold to remove molecules less than 10 kDa and incubated with heparin-agaorse for one hour at 4°C. Conditioned medium from infected fibroblast and choroid smooth muscle cultures was concentrated 10-fold by ultracentrifugation before use. All samples were adjusted for equal protein concentration and 0.2 μm filter sterilized before addition to the cultures.

Shell-less chicken cultures. Plastic tripods were constructed as described by Dunn, 1981[122]. Briefly, 3 inch plastic PVC pipe was cut into the shape of a tripod. A well was made in the tripod using plastic wrap and held in place by a plastic ring made from 3 inch PVC pipe and the whole assembly was UV sterilized for 2 hours and covered with a sterile 100-mm petri dish bottom. E2-E3.5 chick embryos were placed into sterile tripods and

incubated at 38.5°C under high humidity conditions. No antibiotics or calcium were added to the cultures.

Preparation of windowed eggs. Eggs to be windowed were set on their side 12 hours prior to opening and the position of the embryo marked by candling. A small window was cut out of the top of the shell of E3-E4 eggs with the aid of a small hack saw blade and the opening sealed with Blendoderm surgical tape (3M corporation), and incubated for the duration of the experiment on its side at 38.5°C.

RCASBP(A) gene constructs and virus production. The coding region of GPA was subcloned into the SLAX shuttle vector (kindly provided by C. Cepko and Stephan Hughes) and then subcloned into the RCASBP(A) vector. Both constructs were verified by sequencing. Viral stocks were generated by transfecting fibroblast cultures prepared from pathogen-free eggs (Hyvac corp., Adel, IA) with 1 µg RCAS linearized plasmid. Fibroblast cultures were maintained in L-15 supplemented with 10% (v/v) heat-inactivated horse serum (Gibco-BRL) with 33 mM glucose, 50 U/ml penicillin and 50 mg/ml streptomycin. Viral stocks were concentrated by ultracentrifugation at 100,000x g at 4°C for 3 hours. Concentrated stocks were titered by limiting dilution and infectivity of cells measured by staining for p27 gag protein. Only those stocks with titers greater than 10^8 were used in this study and were adjusted to equal number of infectious particles per microliter and stored at -80°C.

Viral infection. Two to four µl of concentrated viral stock was injected directly at multiple sites into the region of the eye of E2.5-3.5 embryos using narrow glass tubing pulled to a fine point. In some instances, a dilute solution of fast green dye was used to assess the efficiency of injection. Concentrated viral stocks were thawed immediately before injecting. DNA injections of purified plasmid DNA was adjusted to 233 ng/ml in

polyethyleimine (PEI) and filter sterilized. For infection of cultured cells, 1 µl of concentrated viral stock (1-7x10^8 infectious particles per ml) was added to each 35-mm or 100-mm plate two days after the original plating of cells.

Counting methods. Ciliary ganglia were isolated from E14 embryos and fixed in 4% paraformaldehyde for at least 72 hours, pre-stained in small amounts of thionin, dehydrated in an ethanol/xylene series and embedded in paraffin. Eight micron sections were cut and floated onto gelatin coated slides. Sections were cleared of paraffin, rehydrated, and subsequently stained with 0.25% thionin, dehydrated and coverslipped. Neuronal profiles were counted by determining the number of neurons with distinct nuclei in every fifth section at 600X in the case of E8 and E11 ganglia and at 400X in every tenth section for E14. Mean cell diameters were calculated by acquiring images with a digital camera and traced within NIH-Image software. The total number of neurons present in the ganglia was calculated by dividing the sum of profiles counted by the number of sections counted, then multiplied by the total number of sections in each ganglion. No correction factor for double counting was made as the total distance between sections was always larger than the largest profile diameter.

Results

Overexpression of chCNTF in chick embryos rescues CG neurons from cell death. Fibroblasts transfected with the either the RCASBP(A)-chCNTF or RCASBP(A)-AP constructs showed prominent staining for p27 gag protein, with virtually all cells showing strong infection (Fig. 1, Panel A). RCAS-AP infected fibroblast cultures showed not only strong infection, but very high alkaline phosphatase activity as well. Cultured smooth muscle choroid cells dissociated from choroid target tissues and subsequently infected with the RCAS-chCNTF virus also showed strong p27 staining and prominent chCNTF-like immunofluorescence (Panel C). By comparison, endogenous chCNTF was undetectable by immunofluorescence (Panel D) and uninfected or cultures infected with vector alone showed no immunoreactivity. Infected choroid cultures had identical morphology, became confluent at the same point, and were as viable as uninfected cultures. Therefore, despite the massive infection with the virus and the high levels of expression of chCNTF, choroid smooth muscle cells were not adversely affected.

CG neuronal survival assays confirmed that much higher levels of chCNTF-like activity were being expressed in transfected fibroblasts in cell culture, with typically 82-fold more biological activity present in either the conditioned medium or from cell extracts (Table 1). The level of biological activity varied considerably between experiments, but always showed substantial elevations in trophic activity. Greater than 80% of this biological activity could be immunodepleted with anti-chCNTF antibodies, verifying the majority of activity being measured was due to chCNTF and not other proteins or molecules with CNTF-like survival activity. The chCNTF-like biological activity was also surprisingly stable. Samples kept at 4°C for two weeks still possessed 50% of the biological activity of fresh extracts. Western blots of extracts from RCAS-chCNTF infected choroid cultures confirmed the full size protein was being made.

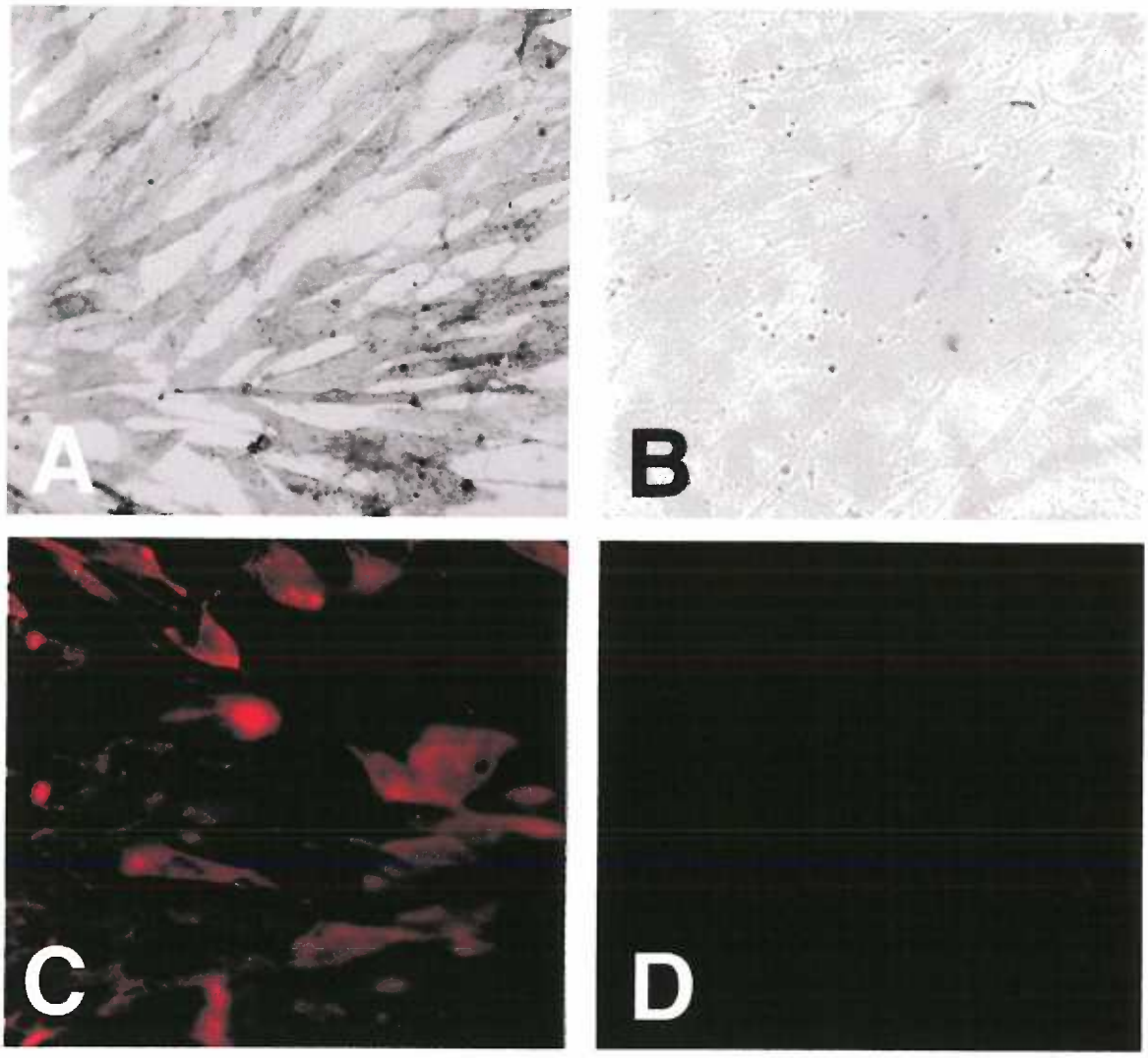

Figure 1. **Infection of chick embryo fibroblasts and choroid smooth muscle cells with RCASBP(A)-chCNTF.** RCASBP(A) was able to efficiently infect cells from emrbyos of a RF7-2 flock of white leghorn chickens, confirming these embryos are very susceptible to infection. Panel *A,* Within 2 days of infection with a concentrated viral stock, nearly every fibroblast stained positive for the viral envelope protein p27 gag, whereas no immunoreactiivty was seen in uninfected control cultures (*B*). Panel *C,* Cultured smooth muscle choroid cells were also efficiently infected as demonstrated by immunofluorescene to p27. Panel *D* shows an uninfected culture. Note: choroid cultures were infected at a lower cell density than in the case of fibroblasts.

Table 1. Overexpression of chCNTF enhances CG survival.

Sample neuronal	Bioassay (trophic units)		Survival
	choroid cultures[a]	eyes[b]	ganglia[c]
Uninfected	n d	nd	3256 ± 851 (n=11)
RCASBP(A)-vector	112 ± 61	455 ± 35	3400 ± 1201 (n=17)
RCASBP(A)-chCNTF	9136 ± 1086	1280 ± 54	5025 ± 1472 (n=29)*

[a]Average of 2 experiments, numbers represent the mean, standard deviations follow.

[b]Average of 2 experiments

[c]Number in parenthesis indicates sample size

* P <0.0001

nd= not done

To facilitate injecting embryos, shell-less chick embryo cultures were prepared [122]. Embryos cultured in this way were healthy and viable and could be maintained through E20, though the viability diminished rapidly after E14. Cell death in the avian ciliary ganglion is largely completed by E14[37]. Embryos placed into tripods at E3, for example, were viable enough to receive injections of concentrated viral stock at one or more sites around the head/eye region by E3.75. To be certain that the development of the embryos was not slowed down nor the development of the targets affected, we compared embryos grown as shell-less cultures and infected with RCASBP(A) with uninfected embryos incubated in the intact egg (Fig. 2). No difference could be seen between the two embryos and the eyes appeared completely normal. Analysis of an E14 embryo infected with RCAS-chCNTF at E4 and found to have 20-fold more biological activity in an eye extract from the uninjected side, showed widespread p27 immunoreactivity throughout both the ciliary muscle and choroid layer of the eye from the infected side (Fig. 3, Panels A and E). Infectivity varied from region to region, but was surprisingly uniform throughout the target tissues examined. ChCNTF-like immunoreactivity was detectable in a similar pattern, although the immunoreactivity levels were lower compared to p27 staining (Panel B and F). Virtually every cell that was immunopositive for p27 also showed immunoreactivity for chCNTF. ChCNTF immunoreactivity was substantially higher than endogenous levels of chCNTF, which is largely undetectable by immunofluorescence at E14 (Panels D and H). Uninfected embryos showed little or no specific p27 immunoreactivity, as would be expected for embryos developing from pathogen-free eggs (Panels C and G). Bioassays of pooled whole eye extracts from different experiments revealed a 1.6-20 fold increase in chCNTF-like activity compared to RCAS-vector infected embryos, with on average a 2.8-fold higher level compared to eyes with viral vector alone (Table 1).

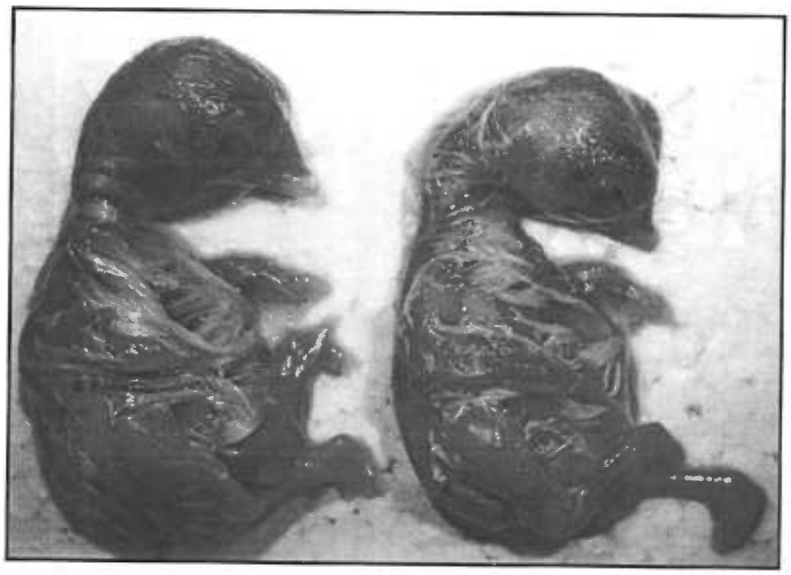

Figure 2. **Embryos grown in shell-less cultures and infected RCAS(BP)A vector developed normally.** Embryo on left was placed into a sterile shell-less tripod at E2.75, infected with RCAS(BP)A-vector at E4 and incubated under high humidity conditions until E14, when it was collected and compared to an uninfected embryo grown in an intact egg (right).

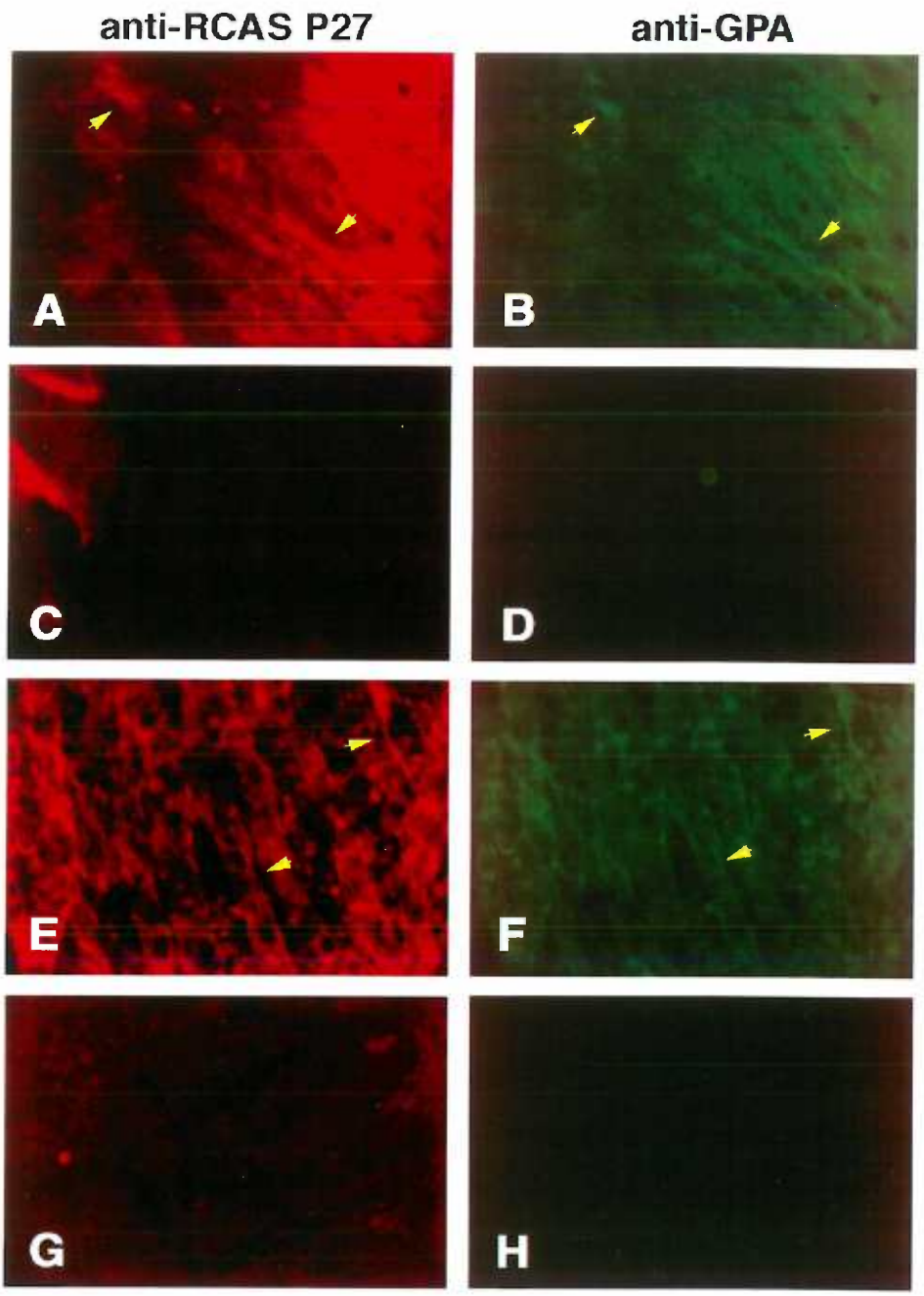

Figure 3. **Overexpression of chCNTF in CG targets *in vivo*.** Shell-less embryos were either left uninfected (*C, D, G*, and *H*) or infected at E4 with RCAS(BP)A-chCNTF (*A, B, E,* and *F*). Isolated iris ciliary muscle (*A-D*) and choroid layer whole mounts (*E-H*) were stained for both p27 gag (*A,C, E,* and *G*) and for chCNTF (*B, D, F,*and *H*). Infected embryos showed very strong p27 immunoreactivity throughout both tissues (*A* and *E*), while uninfected embryos displayed little or no specific signal (the high signal in panel *C* is non-specific fluorescence in the iris). Endogenous chCNTF (*D* and *F*) is barely detectable at E14, but in RCAS(BP)A-ChCNTF infected embryos chCNTF-like immunoreactivity is clearly present (*B* and *F*).

Counts of neuronal profiles from 8 micron thionin stained sections from E14 ganglia collected from embryos infected between E2.5-E4 with RCAS-chCNTF or RCAS-vector showed a 52% increase in the number of neurons in RCAS-chCNTF infected embryos compared to controls (Table 1), which would mean that an additional 23% of the approximately 9000 neurons that would have died off as part of naturally occurring cell death were promoted to survive. A comparison of the two groups of ganglia showed that the CG appeared normal and of about equal size. The difference in neuronal counts could not be explained by the presence of larger neuronal profiles in chCNTF infected embryos, since both average neuronal profile and nuclear profile diameter were the same in RCAS-vector and RCAS-chCNTF ganglia (Table 2). The density of neuronal packing within the ganglia was greater in RCAS-chCNTF infected embryos, as would be predicted if average neuronal size and ganglion size are the same, but the number of neurons is increased (Fig. 4). No obvious effect was seen on neurite outgrowth as both pre- and post-ganglionic nerve bundles appeared normal in cross sections. Embryos infected with concentrated viral DNA containing the chCNTF coding region also showed an increase in CG neuronal survival, compared to embryos receiving vector DNA alone. An examination of E11 CG from embryos infected with the virus showed an identical effect to those examined at E14 (6266, S.D.=636), with 23% more neurons at E11 with respect to E8 levels and normal E11 levels.

To test whether increased numbers of CG neurons at E11 and E14 could be attributed to an increase in formation of neurons prior to cell death, embryos were collected at E8 and the remainder incubated until E14. Ciliary ganglia from E8 RCAS-chCNTF embryos showed normal levels of CG survival. The CG is numerically complete by E6[37, 38] and therefore the neurons present at E8 represent those neurons that have become target-dependent, but have not yet begun cell death.

Table 2. Overexpression of chCNTF does not increase neuronal proliferation.

	Age			
	E8		E14	
property	Vector	chCNTF	Vector	chCNTF
Biological activity (trophic units)	56	220	280	520
CG neuronal counts[1]				
number of profiles	8221± 926	9047 ± 1528	2854 ± 285	4069 ± 78
average cell diameter[2]	18 μm ± 3.7	19 μm ± 1.4	31 μm ± 1.4	33 μm ± 1.3
avg. diameter of nucleus	10 μm ± 0.9	11 μm ± 1.0	17 μm ± 1.3	19 μm ± 1.3

[1]n=3 for all cases

[2]At least 100 neuronal or nuclear profiles were measured in each case.

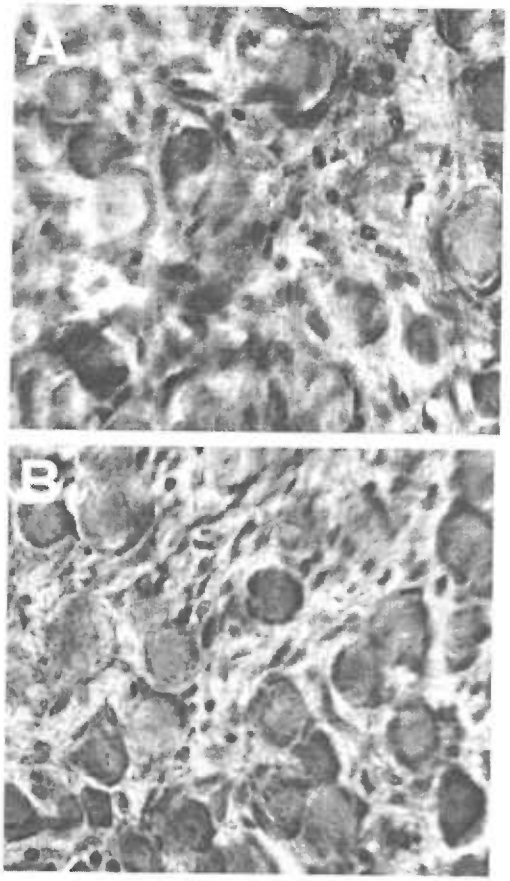

Figure 4. **Overexpression of chCNTF increases neuronal density within the CG.** Thionin stained 8μm paraffin sections from a control RCAS(BP)A-vector infected embryo (Panel *A*) and RCAS(BP)A-chCNTF infected embryo (Panel *B*). Ganglion from normal embryo (*A*) is larger and has a greater density of neurons than ganglion from embryo infected with RCAS virus expressing antisense chCNTF (*B*).

CNTF has a wide range of biological properties in addition to neurotrophic activities. Since it is possible that chCNTF could play multiple roles *in vivo*, it was important to verify that CG targets developed normally. An examination of whole eyes from uninfected, RCAS-vector, and RCAS-chCNTF infected embryos showed that both the overall appearance of the eye and the iris appeared identical (Fig. 5). No morphological differences could be seen in the choroid layers dissected from E14 eyes.

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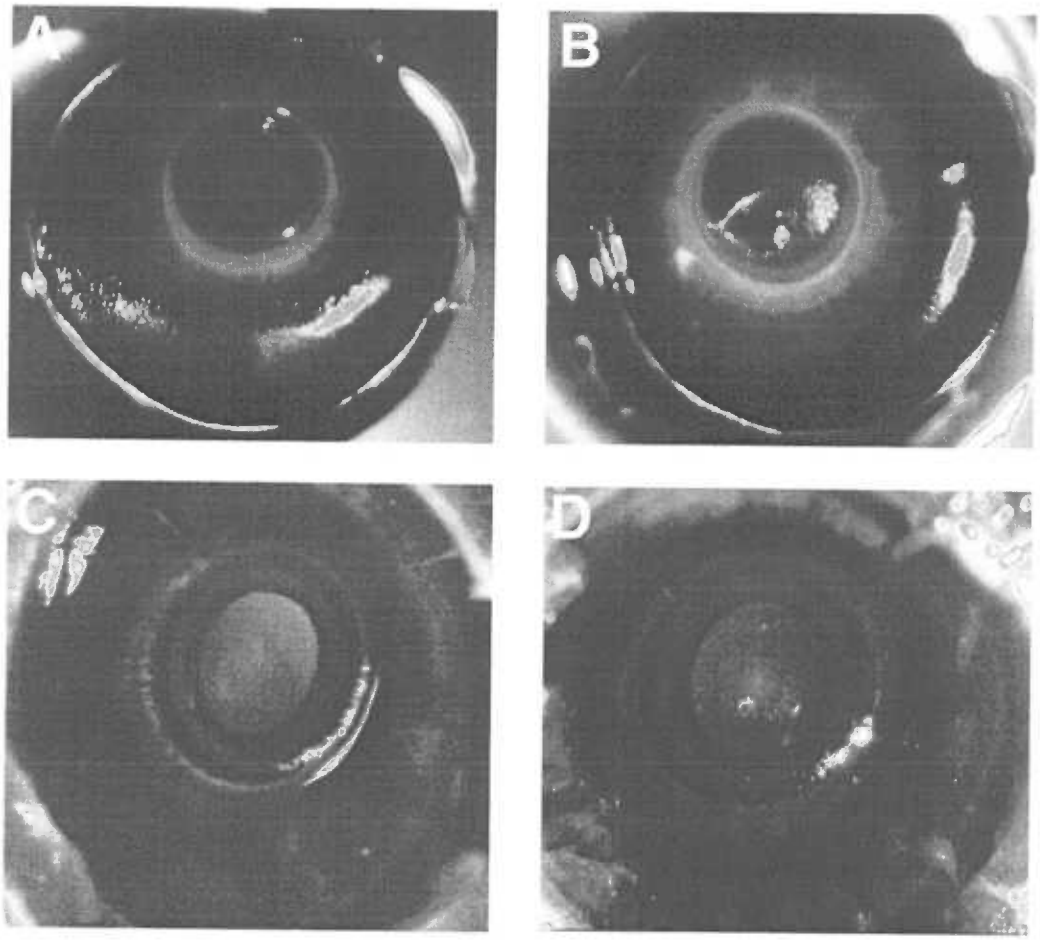


Figure 5. **Development of eyes and target tissues in RCAS-chCNTF infected embryos is unaffected.** Both whole eyes (Panels *A* and *B*) and isolated choroid layers (Panels *C* and *D*) were compared from uninfected (*A*), RCAS(BP)A-vector (*B*), or RCAS(BP)A-chCNTF infected embryos (*C*). Morphologicall, all tissues appear identical. Embryos were infected at E3 and incubated until E14 when they were collected. All tissues were immediately placed in fixative.

Discussion

In an effort to characterize the role that chCNTF may play in regulating CG cell death we have elevated endogenous chCNTF levels using a retrovirus to infect and overexpress chCNTF in chick embryos. We report here that elevation of chCNTF increased by 45% the number of neurons that survived through the end of the cell death phase and that this increase was not due to an increase in proliferation. This enhancement of neuronal survival is important because it not only shows chCNTF can act as a neurotrophic factor *in vivo* as well as *in vitro*, but also suggests that the endogenous factor is limiting- an important criterion for a target-derived trophic factor.

Infectivity and expression of chCNTF via the RCAS retrovirus were effective both *in vitro* and *in vivo*. Infection efficiency of the RCAS virus for both cultured fibroblasts and for choroid smooth muscle cells was very high, with virtually every cell infected within 3 days after addition of the viral stock. Expression levels *in vitro* were much higher than expected, as determined by *in vitro* bioassays, Western blots, and immunocytochemistry. While normally chCNTF produced by choroid cultures is very difficult to detect by even the most sensitive peroxidase-based staining techniques, RCASBP(A)-expressed chCNTF was very easy to detect by immunofluorescence. While RCAS expression in some cases *in vivo* can be non-uniform and too low to be useful, infectivity in this study was quite uniform throughout the eye and expression levels were elevated by a significant margin. *In vivo* RCASBP(A) did not express at nearly the same levels as was seen *in vitro,* but elevations of the factor could clearly be seen by either immunocytochemistry or by measurements of biological activity from target extracts of infected embryos. Elevated chCNTF levels were seen at both the start of cell death (E8) and at the end (E14). Infection appeared to be widespread throughout the targets, although infectivity was not measured for every embryo. One measure of the spread of the virus, however, can be inferred from the observation that eyes from the uninjected side

of the embryo often had elevated levels of neurons as well, and in a few cases was even higher than the injected side. One reason infection was so widespread throughout the eye may have to do with the use of shell-less chick embryo cultures that allowed precise injections of a relatively large amount of concentrated viral stock.

A possible concern when injecting high titer stocks of replication competent virus into early stage chick embryos is the possibility that either the virus or overexpression of chCNTF will adversely affect the embryo, as well as the target tissues themselves. CNTF has been reported to have a wide range of biological properties. CNTF affects neurotransmitter synthesis[123, 124], stimulates the differentiation of type 1 astrocytes[125, 126], and possibly plays a role in neural retinal development[109, 127, 128]. However, we observed no difference in viability of shell-less embryos infected with the virus versus uninjected controls. RCASBP(A)-chCNTF infected embryos developed normally, and the eyes, iris, and choroid layers were indistinguishable from controls- at least at a gross morphological level. The chCNTF receptor is present in brain and neural retina in addition to CG[95]. Therefore, it was possible that these areas could be affected by overexpression of chCNTF. Whole brain, including the cerebellum, appeared unaffected. The lack of a profound effect could be an indication that chCNTF plays very specific roles or that the levels being expressed were too low to have noticeable effects. The possibility of more subtle effects was not examined.

A continual supply of chCNTF through retroviral infection of cells may have been crucial in demonstrating an effect of chCNTF *in vivo*. Application of acidic or basic FGF[129], or CNTF[130] to the chorioallantoic membrane, or direct injection of bFGF[129], or CNTF[130] into the eye, does not lead to enhanced CG survival, even though all of these factors promote survival of CG neurons *in vitro* [53] (however, see Dreyer, et al, 1989)[131]. The lack of an effect of CNTF might be partly explained by the use of human CNTF instead of the chick form which differ 4 to 5-fold in biological activity on CG neurons in culture compared to rat CNTF[131], which is also several-fold lower in biological activity

than chick CNTF[94]. It might also be explained by a requirement of CG neurons for a continuous supply of trophic factor. Assuming some of the injected factor actually reached chick CNTF receptors, this would suggest that a continuous supply of low levels of chCNTF is more important for survival than high concentrations over short periods of time.

The 45% increase in CG neurons seen at E14 represents a rescue of an additional 15% of those neurons at E8 that would have otherwise died as a consequence of naturally occurring cell death. These results are in agreement with those of earlier transplantation studies in which transplantation of an additional optic primordium at E2 as a means of increasing the amount of available target after E8 resulted in an increase in CG neurons by 8-27%[11]. It is unclear why the majority of neurons are not rescued in these experiments. In the case of chCNTF overexpression experiments, it may suggest that other neurotrophic molecules may be involved, which together account for the total target influence. Arguing against this possibility is the observation that nearly 100% of E8 CG neurons can be maintained in culture with purified chCNTF[132]. It should also be noted that in some ganglia as many as 69% of E8 levels of neurons were present. The variation between different ganglia may be a reflection of the variability in infection between embryo to embryo.

One limitation to interpreting these findings is that one does not know whether the effect is solely target-derived or if infection in other areas provides a local source of factor. Since the ganglion is in close proximity to the eye and the embryos were infected with virus at a time when the neurons are still dividing, it is possible, for example, that cells of the CG themselves are making chCNTF. Whether the trophic source is target-derived or locally produced, however, it is still relevant that a single factor can overcome cell death and suggests that trophic levels of this or a combination of factors is what is limiting neuronal survival.

In contrast to the mammalian forms of CNTF, which are considered to be lesion-induced regeneration factors[93, 133], chicken CNTF has properties consistent with being a true target-derived neurotrophic factor for CG neurons. All species isoforms of CNTF lack a traditional N-terminal signal sequence and it is generally considered not to be a secreted molecule[51, 52] or if it is, it is probably secreted at very low levels. Chicken CNTF, on the other hand, has properties very consistent with that of a target-derived trophic factor. Its ability to be readily secreted, probably through an internal signal sequence, its timing, and its distribution in target tissues the neurons innervate are properties which set apart the avian and the mammalian forms of CNTF.

The fact that a complete rescue of CG neurons was not obtained by altering chCNTF levels in this fashion is possibly a function of sub-saturating amounts of trophic factor being expressed and made available to the neurons. Response to trophic factors *in vitro* is only linear in a shallow range around the ED_{50}[53] and rescuing the majority of neurons will likely require much higher levels of expression. For the purposes of this study, however, it was critical to demonstrate that elevating chCNTF levels well within physiologically relevant concentrations can alter the number of neurons that survive. If endogenous chCNTF is indeed a limiting factor regulating CG survival *in vivo*, then CG neurons should be very sensitive to adjustments to those levels.

Chapter 3

Injection of recombinant chicken CNTF *in vivo* rescues CG neurons from lesion induced death.

Thomas P. Finn and Rae Nishi

Abstract

Many neuronal populations undergo a period of death that is believed to be regulated by target-derived factors. Neurons of the chick ciliary ganglion can be supported *in vitro* by chick ciliary neurotrophic factor- a factor known to be expressed in ciliary ganglion targets. If chick CNTF is a true target-derived neurotrophic factor that regulates CG neuronal death *in vivo*, then adjusting its levels *in vivo* should affect the number of neurons that survive. To test this hypothesis, we injected chick embryos with purified recombinant chick CNTF at the start of cell death when the neurons have become dependent upon their targets for survival. Since we did not know how much of the factor would actually reach the neurons or the synapses, we either injected directly into the vitreous of the eye or at the back of the eye near the ganglion itself. Injections were made at a wide range of time points and as single and multiple injections, and for these we analyzed one example of each type of injection that should have yielded the best response to the factor. Embryos injected into the vitreous of the eye at E8, E9, and E10 showed no change in neuronal cell number between saline injected embryos and CNTF injected embryos. CNTF injected into the back of the eye, however, did result in a dramatic difference between the two treatments. In saline injected embryos, 70% of the neurons degenerated in the ganglion on the injected side, presumably due to a lesion generated by the injections. Ganglia from the CNTF injected embryos had showed a restoration back to 116% of normal E14 levels. These results show that CG neurons are very sensitive to chCNTF *in vivo*, at least under lesion conditions, and that even multiple injections of recombinant factor at the beginning of cell death is not sufficient to promote survival.

Introduction

Developmental cell death in the nervous system is a process that is a least partially target-dependent. During development, many neuronal populations undergo a period of cell death whereby the original population is reduced by approximately 50%[4]. The level of neurons that survive can be altered by adjusting the size of the target field the neurons innervate. Ablation and grafting studies have shown that the size of the surviving population correlates with the size of the target field, with more neurons surviving this cell death phase if additional target tissue is grafted on before cell death begins[10] and fewer surviving if the target is ablated[134]. If the same neuronal population is forced to innervate the same target, fewer neurons may survive[15], whereas if part of the same neuronal population is prevented from innervating its target by axotomy, a greater percent survival is seen for the remaining population[8]. Target dependency occurs shortly after neurons have sent processes out to the target and have formed the first synapses. In the chick ciliary ganglion (CG), for example, 50% of both populations of neurons that compose the ganglion die off between E8 and E14[37] and by E8, all the neurons have sent projections to their targets[8]. The targets of CG neurons all reside in the eye and if the eye is removed at before cell death begins, then 90% of the neurons present at E8 die off within a few days [38]. If an additional optic primordium is grafted onto the embryo, up to 27% more neurons survive [11].

One explanation for the target dependency is the production by the target of a limited quantity of factor that the neurons become dependent upon for survival. The neurotrophic theory is based on this interpretation and states that specific factors synthesized by target cells are released at levels too low to support all the neurons within the population, and through a process of target competition some neurons will not receive sufficient amounts of

factor and die by apoptosis. Over the years a number of neurotrophic factors have been discovered, some of which can be localized to target tissues of specific neuronal populations. For the chick CG, the only neurotrophic factor that has been localized so far in CG target cells is chick ciliary neurotrophic factor, also known as growth promoting activity. The extent that chick CNTF regulates CG cell death has yet to be elucidated.

One way to study target-derived factors and their potential to regulate neuronal cell death is to alter the endogenous levels of the factor. For a potential neurotrophic factor to regulate cell death *in vivo*, it must be able rescue additional neurons if its levels are elevated above endogenous levels. One of the most direct ways of altering neurotrophic levels in chick embryos *in vivo* is to perform injections of purified trophic factors. The advantage of injections over transgenic expression approaches is it allows one to adjust roughly the level of factor, to have some control over the location of where the levels will be elevated, and the timing in which the factor is delivered. One also has control over how long the factor is applied or in how continuous of a fashion. Since the trophic dependency of CG neurons *in vivo* is unknown, to CNTF or any other factor, these parameters are important and may have dramatic consequences on the outcome of the survival.

In this study, two types of injections were made: multiple injections into either the vitreous of the eye or behind the eye. Injections into the vitreous of the eye were designed to deliver the factor to where it could diffuse to CG synapses, whereas injections behind the eye should allow access of the factor to the ganglion itself. Injections into the vitreous sometimes caused the eye to deflate slightly, or to be smaller in size, but the ganglion appeared to be unaffected. Injections behind the eye had no detrimental effects on the eye, but massive cell loss was found in the ganglion itself. Vitreal injections showed no statistically significant difference between BSA/saline injected embryos and chCNTF/BSA/saline injected embryos, while embryos injected behind the eye had a complete recovery from the lesion-induced cell death. ChCNTF injections behind the eye did not, however, rescue significant amounts of additional neurons above those that

normally survive through E14. The ability of chCNTF to rescue neurons from lesion induced cell death demonstrates that under these conditions CG neurons can respond to chCNTF *in vivo*. It may also suggest that whatever factors normally are present in the local environment of the CG during the cell death phase, they are insufficient to support survival of CG neurons on their own.

Methods

Shell-less chicken cultures. Plastic tripods were constructed as described by Dunn [122]. Briefly, 3 inch plastic PVC pipe was cut into the shape of a tripod. A well was made in the tripod using plastic wrap and held in place by a plastic ring made from 3 inch PVC pipe and the whole assembly was UV sterilized for 2 hours and covered with a sterile 100-mm petri dish bottom. E2-E3.5 chick embryos were placed into sterile tripods and incubated at 38.5°C under high humidity conditions. No antibiotics or calcium were added to the cultures.

Preparation of windowed eggs. Eggs to be windowed were set on their side 12 hours prior to opening and the position of the embryo marked by candling. A small window was cut out of the top of the shell of E3-E4 eggs with the aid of a small hack saw blade and the opening sealed with Blendoderm surgical tape (3M corporation), and incubated for the duration of the experiment on its side at 38.5°C.

Sample injections. Recombinant chCNTF was injected either directly into the vitreous of the eye, or behind the eye in the vicinity of the eye socket. Recombinant chCNTF was prepared using a polyhistidine-tag vector (Qiagen) as described in Chapter 2, Supplementary section. RchCNTF was diluted from 5 mg/ml frozen stocks into a 1 mg/ml BSA/saline solution to a final concentration of 10 ng/µl. Twenty µl of solution was injected behind the eye or 5 µl of a more concentrated solution into the eye using a 30 gauge needle fitted onto a cotton stuffed P200 micropipette tip attached to a Rainin P200 pipetter. Where possible, different sites behind or into the eye were used for subsequent injections to lessen the chance of lesions forming.

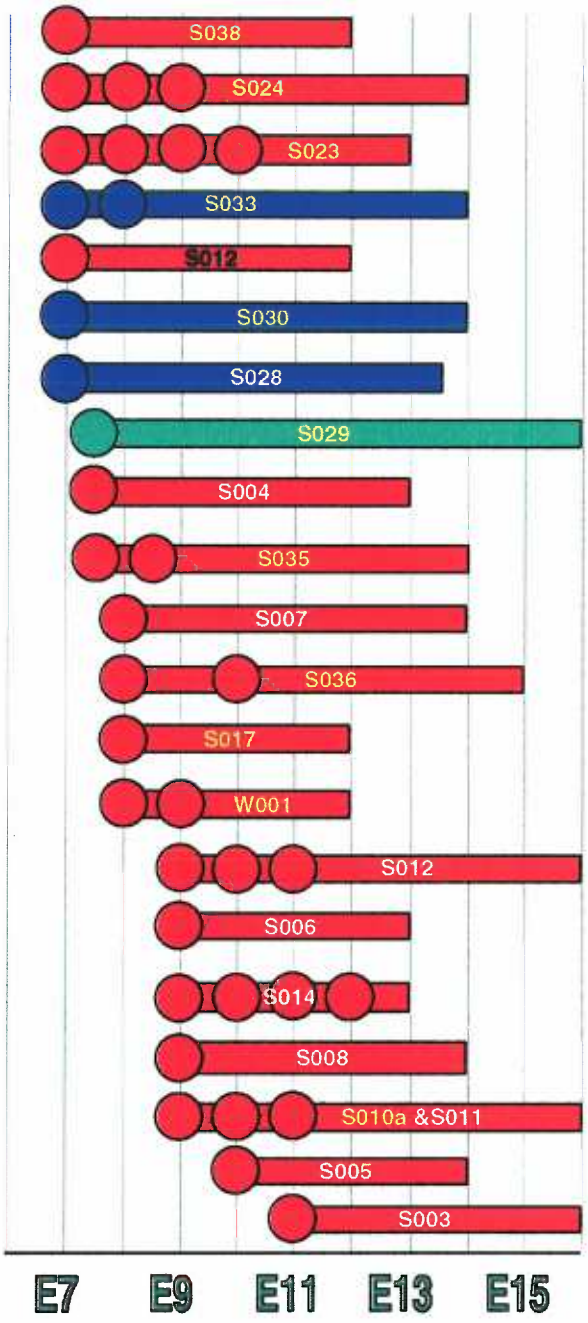
Counting methods. Ciliary ganglia were isolated from E14 embryos and fixed in 4% paraformaldehyde for at least 72 hours, dehydrated in an ethanol/xylene series and embedded in paraffin. Eight micron sections were cut and floated onto gelatin coated slides. Sections were cleared of paraffin, rehydrated, and subsequently stained with 0.25% thionin, dehydrated and coverslipped. Neuronal profiles were counted by determining the number of neurons with distinct nuclei in every fifth section at 600X in the case of E8 and E11 ganglia and at 400X in every tenth section for E14. Mean cell diameters were calculated by acquiring images with a digital camera and traced within NIH-Image software. The total number of neurons present in the ganglia was calculated by dividing the sum of profiles counted by the number of sections counted, then multiplied by the total number of sections in each ganglion. No correction factor for double counting was made as the total distance between every fifth section was always larger than the largest profile diameter.

Results and Discussion

Recombinant chCNTF injected into the vitreous of the eye had no effect on CG neuronal survival. The most direct means of elevating the levels of chCNTF within CG targets is to inject nanogram to microgram quantities directly into the eye (Fig.1). The volume of the eye is occupied by the vitreous, a gel-like material rich in proteoglycans that plays a role in maintaining the shape of the eye. One concern in performing multiple injections into the vitreous is that intraocular pressure might be lost and the targets may deform. Some embryos did show partially collapsed eyes for those eyes that were injected with either saline/BSA or chCNTF/BSA. In some other cases the eye had a normal appearance but were smaller in size and volume. The adverse effects were for the most part a function of the number of injections made. Typically, injections of three or more times into the same eye either resulted in low viability of the embryo and/or smaller eyes. Injections four or more times usually resulted in substantial loss of embryos. Shell-less chick embryo cultures were used throughout these studies because it offered the best way of delivering the most precise injections.

Ganglia from embryos from an experiment in which injections were performed at E7, E8, and E9 were sectioned and counted. The number of CG neuronal profiles was found to be unaffected in chCNTF injected embryos versus controls (Fig. 2). The most direct explanation for these results is that CNTF does not play a role in regulating CG neuronal survival, or if it does, it is not because chCNTF is limiting *in vivo*. This seems unlikely, however, given the fact that chCNTF is present in CG targets, that CG neurons have chCNTF receptors, that chCNTF is a potent trophic factor for CG neurons *in vitro*, and that RCAS overexpression does partially rescue CG neurons from cell death. A more probable explanation is that some aspect of the way in which the factor was delivered was insufficient to promote additional survival. Several possibilities exist: 1) injections at only E8, E9, and E10 may not have sustained chCNTF levels long enough to maintain

chCNTF injection experiments using recombinant chCNTF in vivo



Circles represent dates injected, bars indicate length of incubation from time of first injection

eye socket injection

vitreous injection

anterior chamber injection

Labels on bars indicate experiment number. "S" stands for shell-less experiment, and "W" for windowed eggs. Yellow letters indicate experiments in which at least three or more controls and three or more sample injected embryos survived the full duration of the experiment. White lettered bars are those experiments which had 2 or less survivors in either the controls or samples or both.

Figure 1. List of chCNTF injection experiments performed. About half of all experiments performed had at least three surviving embryo from which to analyze the ganglia. Since the in vivo trophic dependency of CG neurons is not known, and the injection conditions was untested, many experiments were performed to cover a range of possibilities. Injections of recombinant chCNTF were either made behind the eye towards the ganglion, into the vitreous of the eye, or one case into the anterior chamber of the eye.

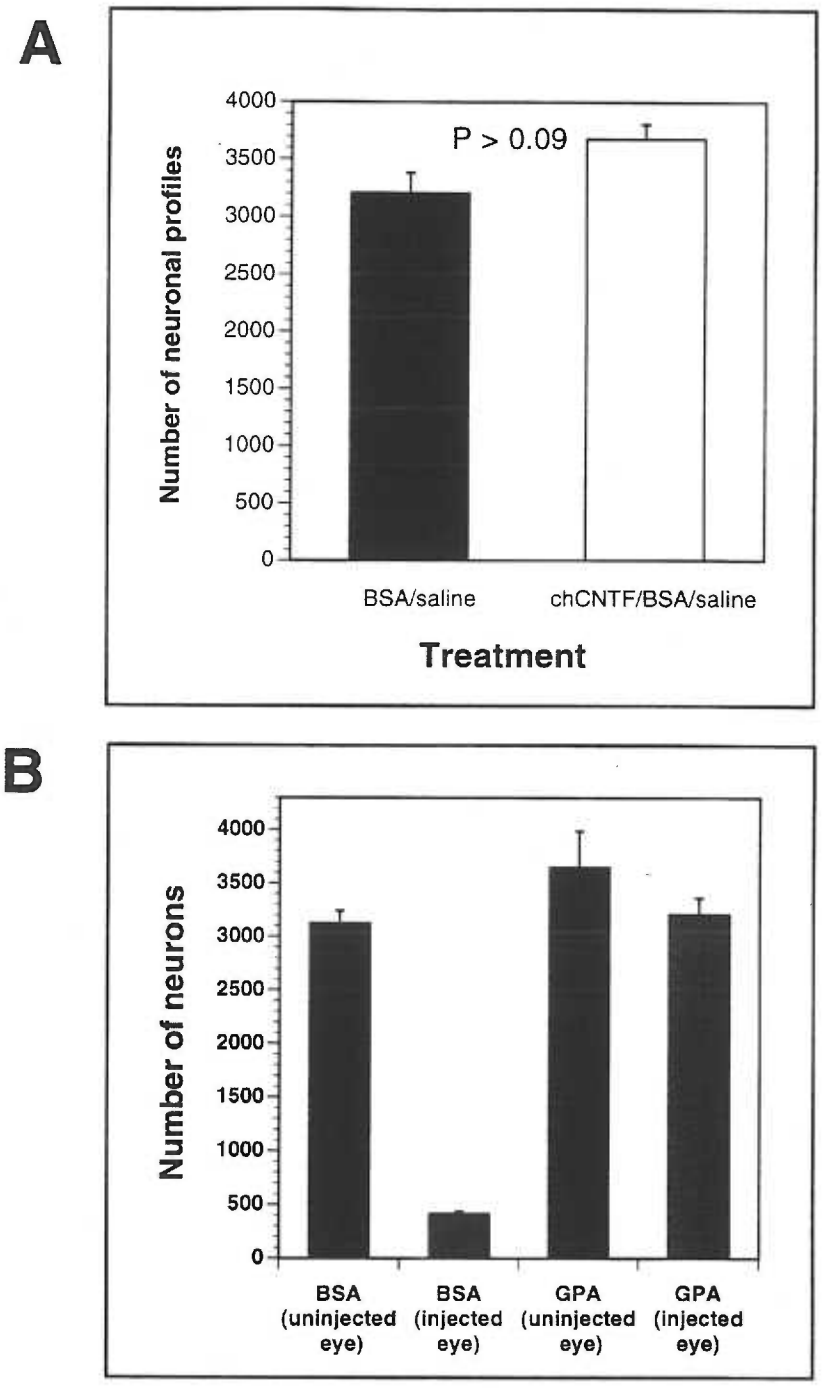


Figure 2. **Chick embryos injected behind the eye with recombinant chick CNTF show enhanced survival under lesion conditions.** Recombinat chCNTF injected into the eye had no statistically significant effect on CG neuronal survival (*A*), but those receiving daily injections of BSA solution into the eye socket of E7 through E9 embryos resulted in elevated CG neuronal death that could be reversed if the solution contained 100ng chCNTF (*B*). n=3 for all cases.

additional neurons. Neuronal counts were performed at E14- had E11 ganglia been examined, an effect might have been seen. E11 ganglia were not counted because the goal of the experiment was to look at neuronal survival at the end of the cell death phase. An effect at E11 might have shown additional survival, but it would be difficult to discriminate between a true survival effect and an effect where CG cell death was merely slowed down. Also, even though CG neurons become less trophic dependent *in vitro* after E13, the trophic requirements *in vivo* are not known. 2) Although the amount injected per eye should represent roughly 1000-fold excess of factor compared to estimated endogenous levels, it is unknown how much of the factor was actually available to the neurons. The majority of the factor may not have penetrated the vitreous and have diffused back out of the eye, or the factor may have been rapidly turned over in the eye. The half-life for CNTF in the bloodstream of rats was estimated to be 2.9 minutes[135]. Since chCNTF receptors are also present in neural retina, some of the factor may have been cleared by binding to neural retina before it continued to diffuse through the pigmented epithelium on its way to the choroid layer. 3) *In vivo* , CG neurons may require a continuous supply of a factor and a large bolus of factor delivered daily may simply be too pulsatile to have an effect. 4) Finally, the lack of an effect might also be explained by a selection process. The embryos that survived multiple injections and the full term of the experiment may reflect those embryos that received/retained the least amount of factor. These explanations could also be applied to the results obtained from eye injections of bFGF and human CNTF that have trophic activity on CG neurons *in vitro* [53, 86, 136], but had no effect on chick CG neurons *in vivo* [129, 130].

Recombinant chCNTF injected into chick embryos behind the eye acts as a lesion factor for CG neurons. One way to possibly circumvent the problem inherent with eye injections, such as sufficient diffusion of the factor to the targets, would be to apply the factor in the vicinity of the ganglion at the beginning of CG cell death. Embryos injected with 100 ng

rchCNTF behind the right eye on E7, E8, and E9 could rescue all of those neurons which died as a result of a lesion created by the injection, but did not significantly enhance neuronal survival over the level normally seen at E14 (Fig. 2). The extent of the lesion was not characterized, but damage to either the afferent or efferent projections could explain the amount of cell death seen[38, 137]. The ability of chCNTF to rescue neurons under these circumstances demonstrates that CG neurons can respond to chCNTF *in vivo*, as was also demonstrated by overexpression of chCNTF using RCASBP(A) (see Chapter 2).

The fact that no additional neurons were rescued by rchCNTF injections over those that normally are found in E14 CG may be an indication that additional factors are required to promote survival of all CG neurons. One must also consider the possibility that the level of chCNTF delivered, though higher than normal physiological levels, may not have reached saturating levels of neurotrophic activity. Since much of the factor likely diffuses away from the site of injection, it is unclear how much of the injected factor remained accessible to the ganglion. Since only one concentration of factor was examined, and since it was not feasible to measure the level of the factor that remained in the area of the ganglion, it is not possible to eliminate this second possibility. Taken together, however, with the previous observation that overexpression of chCNTF in chick embryos can rescue additional CG neurons from cell death, the lesion studies support the hypothesis that chCNTF regulates CG neuronal survival *in vivo*.

Chapter 4

A replication-competent retrovirus as a vector for expression of antisense chCNTF

Thomas P. Finn and Rae Nishi

Abstract

Cell death in the nervous system is a natural process of development. In the avian ciliary ganglion approximately half of the neurons present at E8 die by the end of the cell death phase at E14. The amount of neurons that survive this developmental time window can be affected by the size of the target field made available to the neurons. One influence that the target may have on regulating the amount of neurons that survive is the production and secretion of a target-derived trophic factor. Chicken CNTF is a target-derived factor that has neurotrophic properties both *in vitro* and *in vivo* for ciliary ganglion neurons. We attempted to block endogenous chick CNTF in order to determine if this candidate trophic molecule plays an important role in regulating ciliary ganglion neuronal cell death *in vivo*. Using a replication competent retrovirus as a means to deliver a full length antisense CNTF message, we first tested its ability to block endogenous CNTF in cultured chick choroid smooth muscle cells- a target of ciliary ganglion neurons. CNTF-like biological activity was blocked 65% compared to cultures infected with the viral vector alone. A 55% reduction in chick CNTF-like biological activity was also seen in eye extracts prepared from antisense infected embryos. Despite the reduction in chick CNTF message levels overall, ciliary ganglion neuronal counts of pooled ganglia from six experiments showed no change in the number of neurons present. In three experiments in which trophic levels were known to be reduced, no overall effect was seen in the corresponding ciliary ganglia. In two of these three experiments, however, there was a reduction in the number of neurons between controls and antisense-expressing embryos, but the sample size was too small to establish statistical significance. The lack of an effect overall may be attributed to an inability to reduce CNTF levels consistently and sufficiently, or it may be evidence that other target-derived properties are more important for regulating neuronal survival.

Introduction

The neurotrophic theory attempts to explain how during development many neuronal populations undergo a period of programmed cell death that is regulated by the targets. In many CNS nuclei and PNS ganglia, a developmental time window exits whereby the original neuronal population is reduced by roughly 50%. Neurons begin a specific period of cell death shortly after they have innervated their targets and have become dependent upon them. Regulation of cell death by the targets is thought to be mediated through competition for limited amounts of specific trophic factors secreted by target cells and made available to neurons via high affinity receptors present at the synapse. For some populations a single factor may regulate cell death, whereas in other populations several factors may need to work in combination to support all sub-populations of neurons. While sympathetic neurons seem dependent upon a single factor, spinal cord, DRG, trigeminal ganglia, and facial nucleus neurons may be dependent upon multiple factors. Not only do targets and neurons make multiple factors and receptors, respectively, but some neuronal populations are asynchronous with respect to terminal differentiation of neuroblasts into neurons with cell birth and death occurring at the same time[14, 138, 139]. Given the complexity of neuronal populations and the targets they innervate, discerning which target-derived molecules serve as trophic factors that regulate cell death is difficult to establish.

A relatively simple system by which to study neurotrophic/target dependency is the ciliary ganglion. The ciliary ganglion has only two populations of neurons, both of which are well characterized in the chick. CG ciliary neurons innervate the ciliary body and sphincter muscle of the iris and choroid neurons innervate smooth muscle surrounding blood vessels in the choroid layer of the eye. Chick embryos are especially well suited for CG trophic dependency studies since the ganglion is readily isolated, all the targets are located in the eye, and the eye is accessible throughout development. The chick CG is

numerically complete by E6 and about 90% are target-dependent by the time cell death begins at E8[37, 38]. CG neurons are also one of the few populations that are amenable to culture with high viability- nearly 100% of CG neurons can be supported in culture by a single factor[48, 53].

Of the three groups of trophic factors that have been identified thus far with neurotrophic activity for CG neurons *in vitro*, CNTF is the most likely candidate for a trophic factor regulating CG cell death *in vivo*. Acidic and basic fibroblast growth factors[53], glial derived growth factor (GDNF)[119] and ciliary neurotrophic factor (CNTF)[53] all support CG neurons in culture, though to varying degrees of potency. *In vitro*, chick CNTF (chCNTF) purified from embryonic eye supports CG neurons in long term culture with an ED_{50} of 12 pg/ml[53] and recombinant chCNTF has similar potency and can maintain cultures for at least three weeks[120]. ChCNTF message is present in chick eye as early as E6[54], chCNTF-like trophic activity is readily detectable at E9, and chCNTF-like immunoreactivity is detectable in at least one of the targets by E10[53], ChCNTF-like immunoreactivity is present in target cells of both choroid and ciliary neurons, and chCNTFRα message is present in the CG as early as E6[54]. Finally, elevation of chCNTF levels *in vivo* enhances CG neuronal survival (see Chapter 2).

To fully establish CNTF as a true target-derived trophic factor, it will be necessary to show that reduction in endogenous chCNTF levels reduces the amount of CG neurons that normally survive. In this study we report that chCNTF-like biological activity can be reduced 54% *in vivo* through the use of a retroviral delivery system. Reduction of chCNTF to this degree under the experimental conditions described here, did not however, significantly reduce CG neuronal survival. The results were highly variable, partially due to the fact that the total cell number in normal CG differs significantly, and viral infection may have varied considerably from embryo to embryo. Additional experiments will have to be performed under conditions where large reductions in

chCNTF levels are known to have occurred in target tissues in individual embryos and the corresponding ganglia analyzed.

Methods

ChCNTF bioassays. Short term (2-5 days) CG neuronal survival assays were performed according to Eckenstein, et al., 1990[53] using E8 (Stage 34) ciliary ganglia, except that ganglia were plated at a density of 0.75 ganglia per well in 48-well plates. Neuronal survival was determined by counting the number of large, phase-bright cells with processes greater than 3 cell diameters in 4 day cultures. Sixteen fields of view were counted per well and all samples were tested in duplicate or triplicate.

Preparation of extracts. Fibroblast or choroid smooth muscles cells were collected from two 100-mm plates, pelleted by centrifugation, then resuspended in a small volume of 10 mM NaMOPS, 1 mM EDTA pH 7.4 with a cocktail of protease inhibitors. Extracts were prepared by sonication for 45 seconds on ice with a probe type sonicator and the lysate centrifuged at 30,000 x g for 20 minutes at 4°C. Eye extracts were prepared by a similar protocol, except samples were adjusted to 0.5 ml buffer for each eye used and homogenation with a Teckmar tissue homogenizer for 45 seconds on ice prior to sonication. All lysate supernates were concentrated by ultrafiltration 10-fold to remove molecules less than 10 kDa and incubated with heparin-agaorse for one hour at 4°C. Conditioned medium from infected fibroblast and choroid smooth muscle cultures were concentrated 10-fold by ultracentrifugation before use. All samples were adjusted for equal protein concentration and 0.2 μm filter sterilized before addition to the cultures.

Preparation of windowed eggs. Eggs to be windowed were set on their side 12 hours prior to opening and the position of the embryo marked by candling. A small window was cut out of the top of the shell of E3-E4 eggs with the aid of a small hack saw blade and the opening sealed with Blendoderm surgical tape (3M corporation), and incubated for the duration of the experiment on its side at 38.5°C.

Shell-less chicken cultures. Plastic tripods were constructed as described by Dunn [122] Briefly, 3 inch plastic PVC pipe was cut into the shape of a tripod. A well was made in the tripod using plastic wrap and held in place by a plastic ring made from 3 inch PVC pipe and the whole assembly was UV sterilized for 2 hours and covered with a sterile 100-mm petri dish bottom. E2-E3.5 chick embryos were placed into sterile tripods and incubated at 38.5°C under high humidity conditions. No antibiotics or calcium were added to the cultures.

RCASBP(A) gene constructs and virus production. The coding region of GPA was subcloned into the SLAX shuttle vector as described in Chapter 2, except it was inserted in the reverse and complimentary orientation.

Viral infection. Concentrated viral stock was injected directly at multiple sites into the eye region of E2.5-3.5 embryos using narrow glass tubing pulled to a fine point. In some instances, a dilute solution of fast green dye was used to assess the efficiency of injection. Concentrated viral stocks were thawed immediately before injecting. DNA injections of purified plasmid DNA was adjusted to 233 ng/ml in polyethyleimine (PEI) and filter sterilized. For infection of cultured cells, 2-4 µl of concentrated viral stock ($1\text{-}7 \times 10^8$ infectious particles per ml) was added to each 35-mm or 100-mm plate two days after original plating of cells.

Counting methods. Ciliary ganglia were isolated from E14 embryos and fixed in 4% paraformaldehyde for at least 72 hours, dehydrated in an ethanol/xylene series and embedded in paraffin. Eight micron sections were cut and floated onto gelatin coated slides. Sections were cleared of paraffin, rehydrated, and subsequently stained with 0.25% thionin, dehydrated and coverslipped. Neuronal profiles were counted by determining the number of neurons with distinct nuclei in every fifth section at 400X in the

case of E8 through E11 ganglia and in every tenth section for E12-E20. Mean cell and nuclear diameters and areas were calculated by acquiring images with a digital camera and tracing within NIH-Image software. The total number of neurons present in the ganglia was calculated by dividing the sum of profiles counted by the number of sections counted, then multiplied by the total number of sections in each ganglion. No correction factor for double counting was made as the total distance between sections was always larger than the largest profile diameter.

Results

RCAS-antisense chCNTF reduces endogenous chCNTF levels. As a means of testing the ability of antisense message to chCNTF to effectively block production of endogenous chCNTF, we tested both infected cultured target tissues as well as eyes from infected embryos for a reduction in chCNTF-like biological activity. Such constructs were prepared by inserting the coding region of chCNTF in the reverse orientation so that a complimentary transcript would be made. For the *in vitro* studies we prepared cultures of one of the natural targets of CG neurons, choroid smooth muscle cells, and infected them with either RCASBP(A) virus or the antisense-chCNTF expressing RCASBP(A). Choroid smooth muscle cultures normally showed a several-fold increase in chCNTF message at day 6 of plating. The ability of RCAS-antisense chCNTF to block the rise in expression was tested by measuring chCNTF-like neurotrophic activity on CG neurons *in vitro* from either cell extracts or choroid conditioned medium. Extracts and conditioned medium from 8 day cultures showed an average reduction in chCNTF-like biological activity of 60% in RCAS-antisense chCNTF infected cultures versus those infected with viral vector alone (Table 1). The effect was very reproducible and similar reductions in biological activity were seen in 20 day cultures. RCAS chCNTF-antisense infected choroid cultures grew at the same rate and had the same morphology as uninfected cultures, therefore, differences in biological activity were probably not due to differences between the cultured cells themselves.

To test the ability of the RCAS-antisense chCNTF to block endogenous chCNTF in CG targets in the eye we infected E2-E4 chick embryos with concentrated viral stock, then measured the biological activity in eye extracts from pooled E14 chick eyes. To allow for more precise delivery of the virus near the eye, shell-less chick embryo cultures were prepared (see Appendix 2). Embryos infected with either the virus alone or with the

RCAS(BP)A-antisense chCNTF expressing virus showed no overall effect on the viability or health of the embryos or on the development of the eye, at least at a gross morphological level. Extracts from pooled E14 RCAS-antisense chCNTF infecting eye did show approximately a 60% reduction in chCNTF-like biological activity (Table I), in close agreement with results obtained with choroid smooth muscle cultures.

Expression of antisense chCNTF in vivo reduces CG neuronal survival. Profile counts from CG paraffin sections revealed a small decrease in the level of CG neurons in E14 CG from embryos infected with the antisense expressing virus vs. viral vector alone. The level of decrease varied considerably. Part of this variation could be due to the natural variance in the size of the neuronal populations as both control ganglia and ganglia from untreated embryos showed significant differences in the total number of neuronal profiles at E14 (see also Appendix, Section 7.2). A total of six experiments were performed and the outcome of these experiments is given in Table 1. From these experiments a total of 34 ganglia were collected from RCAS-vector controls and 26 from RCASBP(A)-antisense chCNTF infected embryos. Pooling the data from these seven experiments together showed no significant difference between any of the groups as measured by a two-tailed t-test. In three of these experiments the level of biological activity in eye extracts was determined. Two of these experiments showed a small, though not statistically significant, difference in the number of neurons present in the ganglia in antisense infected embryos versus controls.

Table 1. Overexpression of antisense chCNTF reduces chCNTF-like activity *in vitro* and *in vivo*, but does not affect CG neuron survival.

	Sample		
Bioassay (trophic units)	**Uninfected**	**RCAS vector**	**RCAS vector + antisense-CNTF**
choroid cultures (n=6)	ND	915 ± 665	326 ± 246
eye extracts (n=3)	ND	520 ± 160	243 ± 116
neuronal survival			
CG neuronal counts (number of profiles)	3461 ± 1209 (6)[a]	4108 ± 1209 (17) (uninj)[b] 4196 ± 1025 (17) (inj)	3865 ± 982(17) 3865 ± 1261(9)
Neuronal profiles			
average cell area[c]	572 μm^2 ± 102	581 μm^2 ± 161	565 μm^2 ± 84
average cell length	34.6 μm ± 4.7	34.2 μm ± 5.2	34.2 μm ± 2.5
avg. area of nuclei	182 μm^2 ± 40	177 μm^2 ± 49	170 μm^2 ± 24
avg. length of nuclei	19.4 μm ± 3.2	19.1 μm ± 2.8	19.3 μm ± 1.5

[a] Numbers in parenthesis indicate sample size.
[b] Value is for pooled ganglia from uninjected side of embryo.
[c] At least 100 measurements were made in each case.

Discussion

For chick CNTF to be established as a target-derived trophic factor that regulates CG neuronal cell death during development, demonstration of *in vitro* trophic activity on CG neurons, localization of chCNTF at the correct time and place in development, and demonstration that modulations of chCNTF levels *in vivo* have a significant effect on CG neuronal survival must be shown. ChCNTF meets all the descriptive requirements for a trophic factor: it is present at the appropriate time and location in CG targets during development to play a role in CG survival and it is a potent neurotrophic factor for CG neurons, even under single cell conditions. Experimental evidence comes in the form of overexpression of chCNTF in chick embryos and injections studies using recombinant chCNTF. In the case of the former, additional CG neurons survive if chCNTF is expressed in the targets during that time. Recombinant injections have shown that chCNTF can rescue a large percentage of those neurons that die under lesion conditions brought about by the injections.

The remaining criterion to establish chCNTF as a target-derived neurotrophic factor is demonstrating CG neurons require this factor to survive and that under conditions where the endogenous factor is lowered, fewer CG neurons survive. In this study, we show that endogenous chCNTF can be lowered using an antisense message expressed using an replication competent retrovirus, and that this holds true both *in vitro* and *in vivo*. The level of the block is significant, 65% *in vitro* and 54% *in vivo*. It would no doubt be advantageous to have an antisense block that was more complete, and it is possible that shorter stretches of the coding region or of 5' upstream sequence would yield a better block of chCNTF translation or decreased message stability. However, synthetic oligonucleotides designed and tested by others to interfere with chCNTF production showed only a marginal effect *in vitro* (data not shown). Furthermore, since CG neurons should be very sensitive to trophic factor levels *in vivo*, a block of 50-60% chCNTF-like

activity is very significant. CG neuronal counts in two experiments did show a reduction in CG survival in antisense-expressing embryos compared to controls, but overall values from pooled experiments showed no reduction in the level of CG neurons between the two conditions.

Demonstration of the requirement that CG neurons have for this factor was partially obscured by the high levels of variability in neuronal counts. Variability between different ganglia can be attributed to several factors. First, the number of neurons in control, uninfected E14 ganglia was found to be considerable. The variations in controls was not unique to this strain of chicken as counts from a large number of other CG from another strain at different developmental ages showed similar levels of variability (see Appendix 7.2). Such differences are not surprising considering all the possible differences from embryo to embryo for such parameters as starting numbers of CG neurons, the extent of innervation, the amount of target cells present, the number of synaptic branches, rates of secretion of target-derived factors, etc. These variations made it impossible, however, to assign statistical significance across data sets from pooled experiments, given the small size of the data sets. Also, given the high standard deviation in any particular data set, it was estimated that a population sample size of 16 for each condition would have to be obtained to achieve a p value less than 0.05. Obtaining such a sample size is difficult, though feasible, given limitations in setting up large scale shell-less experiments and the relatively low viability (typically less than 50%) of embryos surviving through E14. Collecting embryos at E12, where viability is greater could help offset this problem.

A second source of variability is undoubtedly the spread of the retrovirus in chick tissues. While the spread of the virus was not determined for each embryo, conditions had been optimized to get strong expression in CG targets. To better analyze all the data collected from neuronal counts, it is advantageous to have a measure of the level of spread of the virus, quantitative biological assays of CNTF-like activity being the best measure.

While it is difficult to draw a clear conclusion from these experiments, it does suggest that the decrease in survival of CG neurons *in vivo* correlates with a greater decrease in biological activity in the eye. Although pooled data sets show no overall difference, the observation that two individual experiments did show a measurable, but not statistically significant difference, suggests that under the right conditions, reduction of chCNTF can reduce CG survival. To make a firm conclusion with these types of experiments will require performing the experiments on a larger scale to obtain a larger sample size, and to separate out those embryos that were poorly infected. Therefore, we can neither say that data from these experiments either support or disprove the hypothesis that chCNTF regulates CG cell death *in vivo*. The fact that a few individual data sets did achieve statistical significance is encouraging and warrants further investigation.

Discussion

6.1. Summary of results.

In this study, I have characterized chCNTF and developed reagents to test the hypothesis that chCNTF is a target-derived trophic factor for CG neurons. This study was essentially organized into three phases: first, a recombinant form of chCNTF was prepared by three different protocols and used to characterize the factor *in vitro* and to study CG neurotrophic dependency *in vitro*; second, the developmental expression and localization of chCNTF was determined by quantitative bioassays and immunocytochemistry using monoclonal and polyclonal antibodies to recombinant chCNTF, and by Northern blot analysis using probes specific for chick CNTF; third, the ability of chCNTF to modulate CG neuronal survival was tested by either direct injection of chCNTF into chick embryos or by expression of chCNTF using a replication competent retrovirus. An attempt was made to reduce endogenous chCNTF levels using a replication competent retrovirus to overexpress antisense chCNTF.

From the first phase, I found recombinant chCNTF to be a potent neurotrophic factor *in vitro*, both from the respect of the native molecule (ED_{50} 1-30 pg/ml and 12 pg/ml, respectively) to recombinant forms of CNTF from other species (30-200 pg/ml) [72, 88, 140, 141]. Recombinant chCNTF is remarkably resistant to denaturation by SDS, and has a tendency to aggregate in the absence of added detergents. Although not predicted from hydrophobicity plots, chCNTF was found to be considerably hydrophobic. CG neurons can be maintained for weeks in medium supplemented with 1ng/ml recombinant chCNTF. CG neurons are dependent upon the factor at the beginning of the cell death phase, i.e. E8, as virtually 100% of the neurons die off within 48 hours in culture in the absence of the factor. By E14, however, CG neurons have become much less dependent

upon the factor and the role of chCNTF at this age and into adulthood may serve other purposes.

From the second phase, I generated antibodies specific for chCNTF and used them to localize chCNTF within structures of the eye and sciatic nerve. Chick CNTF localizes with the same immunoreactive pattern as rat CNTF in rat sciatic nerve, i.e. to the cytoplasm of Schwann cells surrounding nerve fibers, although the staining intensity was much weaker and far fewer cells were positive for immunoreactivity. Within the eye, chCNTF localizes to smooth muscle fibers in the choroid layer and to striated muscle fibers in the ciliary body adjacent to the iris, both of which are natural targets of CG neurons. ChCNTF-like immunoreactivity was also detected in retinal ganglion cells in the neural retina and Northern blot analysis confirms the presence of chCNTF message in neural retina, which differs from previously reported results[54], but is in agreement with the localization of chick CNTFRα. ChCNTF's developmental expression can first be detected by immunocytochemistry at E10. Quantitative bioassays show that chCNTF levels remain low during the cell death phase, but increase more than 10-fold thereafter. Northern blots were used to identify other areas of the embryo where chCNTF may be expressed. Weak signals were detected in pectoral or leg muscle, while neural retina, cerebellum, and brain were found to contain as strong of signal as whole eye or sciatic nerve.

From the third phase of the project, I found chCNTF can rescue a large percentage of the neurons that die as a consequence of an injection-induced lesion. Under non-lesion conditions where the factor is expressed through a replication competent retrovirus, chCNTF can rescue on average 23% of those neurons that would otherwise have died as a consequence of naturally occurring cell death. The total amount of survival between ganglia from embryos infected with the overexpressing construct varied considerably, with the maximum amount of survival at 72%.

6.2 Relevancy of findings.

6.2.1. Characterization of recombinant chick CNTF and trophic dependency of CG neurons in vitro. The importance of demonstrating biological activity of purified molecules is twofold: first, it shows that a given molecule is necessary and sufficient to support neuronal survival in the absence of all other target-derived effects, and 2) it establishes the potency at which the factor functions to override programmed cell death. The latter is critical, because at high concentrations some proteins, lipids, metabolites, etc. may slow down cell death and molecules in the same biological class may bind with low affinity to endogenous receptors. At physiologically relevant concentrations, however, only true neurotrophic factors will support survival, either short term (several days), or in long term cultures (several weeks). In the 250 ng/ml, LIF, a molecule with very similar 3-D structure to CNTF, can bind to the CNTF receptor complex and will support CG neuronal survival *in vitro*. However, at more relevant concentrations of 5 ng/ml, LIF will not support survival for even 24 hours (Finn, unpublished observations).

Demonstrating the same biological activity of recombinant purified factor is also important. Considering the very potent nature of neurotrophic factors and their extremely low abundance, it is possible that contaminating proteins present in very low levels in purified factor preparations could be responsible for some or even all of the trophic activity. Establishing the trophic properties of a recombinant form verifies the activity is really due to the protein in question. Once a factor has been expressed and purified it can be used to characterize both the biological properties of the protein as well as screen other neuronal populations for sensitivity to the factor.

Like other CNTFs, chicken CNTF is remarkably resistant to inactivation by denaturing detergents such as SDS. In fact, all detergents tested upon chCNTF increase its biological activity. This may be partially due to chCNTF's strong tendency to aggregate, especially at high concentration conditions used for storage. Recombinant chicken CNTF typically had an ED_{50} between 1 and 30 pg/ml depending on the

preparation, the expression vector, and the purification protocol. Recombinant forms of CNTF from other species, by comparison, has less biological activity on CG neurons, probably due to differences in purification techniques as well as poorer receptor binding and activation due to species differences in the way the proteins fold. ChCNTF-like activity from extracts of chick eyes and choroid layers is stable for several weeks at 4 °C.

Recombinant chCNTF was used to characterize the trophic requirements of CG neurons *in vitro*. CG neurons do not require any factor for the first 6 hours after plating and proceed to extend numerous neurites. Within the next 12-36 hours virtually all of neurons cultured in the absence of added factor die off, but those cultured in the presence of 1 ng/ml chCNTF can be cultured for several weeks. No obvious effects on neurite outgrowth have been observed. While E8 neurons have an absolute requirement for the factor, E14 and E16 neurons seem far less dependent on the factor. Only a subtle difference could be seen in E14 or older neuronal cultures in the presence or absence of factor. This held true even when neurons were plated in isolated wells by limiting dilution. Therefore, if non-neuronals supply chCNTF to CG neurons *in vivo* at later stage of developments, the factor may be performing a function different than that of a trophic factor.

Recombinant chCNTF was also used to help identify and characterize the chCNTF receptor as part of a collaboration with other groups. The CNTF receptor is a multisubunit complex. It is composed of the 68 kDa CNTF receptor alpha subunit(CNTFRα along with one LIF receptor beta subunit (LIF-Rβ) and GP130 forming a 250 kDa protein complex (Fig. 1). CNTFRα is a glycosylphosphatidyl-inositol-linked protein and therefore does not span the plasma membrane. LIF-Rβ and GP130 are both membrane-spanning proteins, but neither has kinase activity. Instead, ligand binding transduces a signal by changing the receptor's conformation and allows phosphorylation via Jak kinases which bind to SH-2 domains on the intracellular portions of LIF-Rβ and GP130.

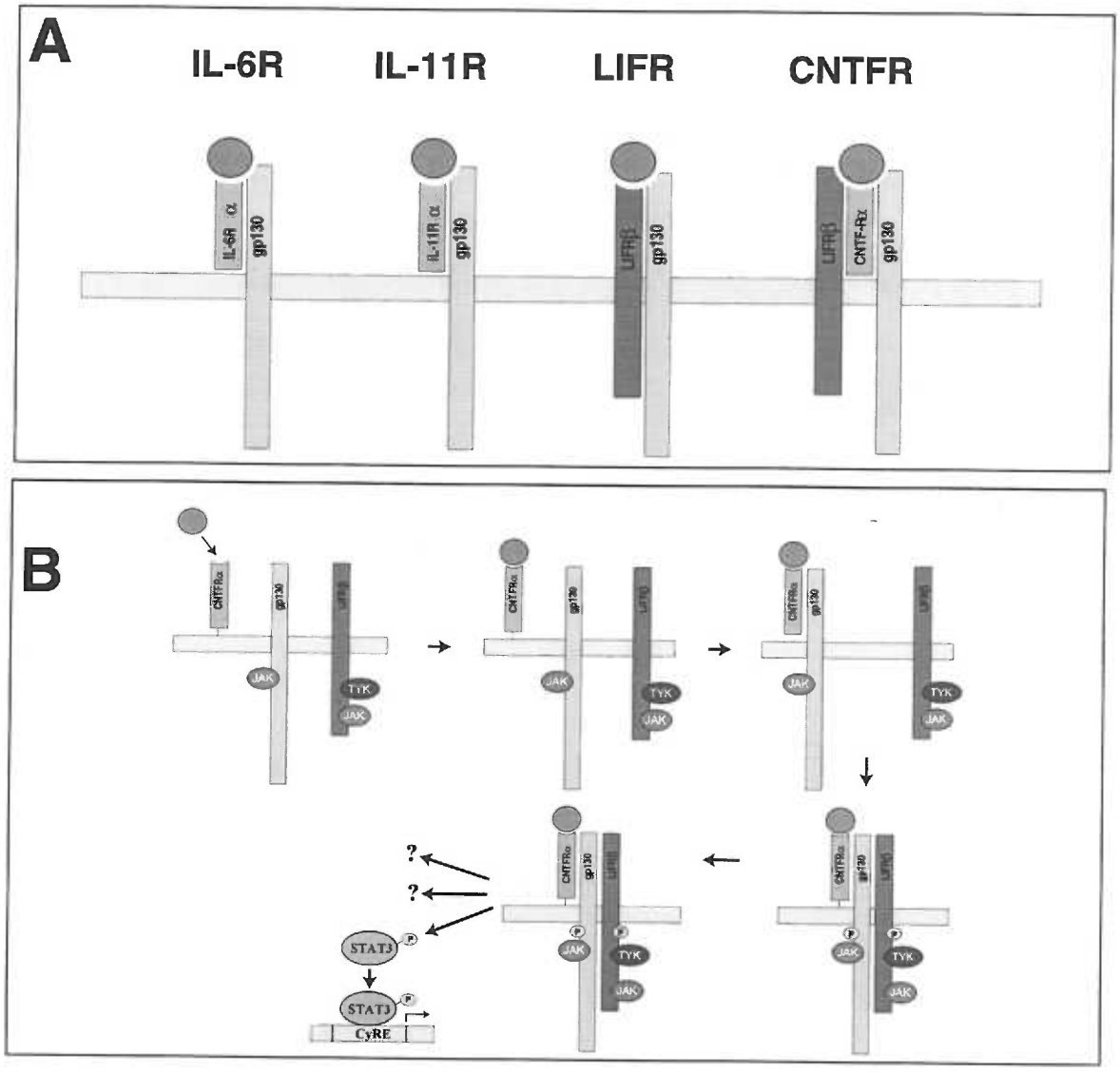


Figure 1. **IL-6 cytokine receptor superfamily structure and signalling.** Panel *A*, all members of the family use the transmembrane protein gp130, at least partially, to transduce a signal across the membrane, while using an accessory membrane bound protein to generate specificity in binding of ligand. The CNTF receptor is composed of CNTFRα and shared components of the IL-6 and LIF receptors. The IL-6Rα, IL-11Rα and CNTRFα ligand binding subunits are linked to the membrane via glycosyl-phosphatidylinositol linkages. Ligand binding causes association with gp130. Panel *B*, sequence of CNTFR activation. Binding of CNTF allows gp130 to associate with CNTFRα which then incorporates LIFRβ as part of the complex. In this context, LIFRβ substitutes for the normal formation of a gp130 homodimer as found in activated IL-6 and IL-11R complexes and mimics LIFRb/gp130 heterodimers in the case of the activated LIFR. Formation of a ternary complex leads to activation of prebound JAK and TyβK kinases that phosphorylate gp130 and members of the STAT family of transcription factors. Phosphorylated STAT then translocates to the nucleus where it binds to cytokine response elements and likely other sites.

Activation of the Jak kinases leads to phosphorylation of Stat-3 which translocates to the nucleus and activates gene transcription.

Several lines of evidence establish that chCNTF is a secreted molecule. First, CNTF-like activity is detectable from conditioned medium of choroid smooth muscle cells under culture conditions where very little cell lysis has taken place[54]; second, chCNTF transfected into COS cells is detectable in conditioned medium, while rat CNTF transfected into an identical vector into sister cultures is not[54]; third, CNTF-like activity is present in conditioned medium of chick embryo fibroblasts infected with RCAS over-expressing chCNTF, but not in similar cultures infected with the virus alone. This activity can be immunodepleted with rabbit anti-chCNTF polyclonal antibodies. The ability of chCNTF, which lacks a traditional N-terminal signal sequence, to be secreted is probably due to the presence of a 20 amino acid internal signal sequence 40 amino acids downstream from the N-terminus. In direct comparison of conditioned medium and cell extracts, typically 50% of the total biological activity is present in the cellular fraction.

6.2.2 Localization in targets and other tissues. At the time this project was started, it was already known that chCNTF message was present within chick eye, sciatic nerve, and the choroid layer of the eye[54], and chCNTF protein had been purified from adult sciatic nerve and E15 eye[54]. It remained to be demonstrated that within the eye chCNTF localizes to the target cell types the neurons innervate. It was possible, for example, that myelinated fibers could have been the source of chCNTF in the eye. As part of this project, I demonstrated that chCNTF localizes to both the striated ciliary muscle fibers and choroid smooth muscle cells the ciliary and choroid neurons innervate, respectively.

The expression of chCNTF message and protein was completely consistent with its potential role as a target-derived neurotrophic factor. Though chCNTF-like immunoreactivity could not be detected until E10 (1-2 days after cell death has begun), chCNTF message levels could be detected as early as E6, and chCNTF-like biological

activity was readily detectable at E9. The discrepancy between the two protein techniques can be explained both by the extreme sensitivity of the bioassay versus immunocytochemical techniques and the fact that at E10 very little smooth muscle is present in the choroid layer. Expression within the choroid layer appeared to be limited exclusively to smooth muscle cells- even myelinated sympathetic fibers that course though the choroid layer were negative for immunoreactivity. ChCNTF was, however, found to be expressed elsewhere in the eye. Northern blot analysis showed a distinct signal in neural retina. Immunodepletion studies of eye extracts show that about half of the CNTF-like trophic activity present in the eye was due to chCNTF or CNTF-like molecules. This finding was important because the eye is rich in trophic factors and if chCNTF represented only a minute fraction of the total biological activity present in the eye, it may have questioned the relevancy of chCNTF as a regulator of CG cell death.

It would have been valuable to screen the CG itself for chCNTF message and protein. The CG, however, is a very small tissue and each embryo contains only two ganglia. While it would be possible to perform PCR analysis on pools of CG, Northern blots and RNAse protection assays are probably not feasible. The chCNTF polyclonal antibodies generated for use in this study do not stain for chCNTF on sections mounted to slides and E10 and younger CG are very difficult to work with as floating sections. Regardless, the possibility that satellite cells provide a local trophic source for CG neurons during the first few days of naturally occurring cell death is unlikely, as the number of satellite cells in a CG is very small at E8.

The expression of chCNTF message in brain, including cerebellum, and in neural retina, suggests that chCNTF may regulate neuronal survival of other populations of neurons. CNTF can support survival of rat purkinje cells[105], hippocampal neurons[111], and retinal ganglion cells[107]. ChCNTFRα expression patterns are consistent with this idea and also suggest a role for chCNTF in the differentiation of sympathetic neurons[95, 124] and photoreceptor rod cells[123, 127].

6.2.3. Modulation of CG survival in vivo with chCNTF. An advantage to studying neuronal survival in the ciliary is that the generation of post-mitotic neurons is completed prior to the onset of neuronal cell death. Sympathetic ganglia, sensory ganglia, and retinal ganglion cells, for example, have neurons being born at the same time other neurons are dying[14, 138, 139]. Not only does this make it difficult to estimate the magnitude of overall cell death, but it is also difficult to examine any potential effect raising or lowering a trophic factor may have on cell death at a given time point. In the avian ciliary ganglion neuronal proliferation is complete by E6[37], and both populations of CG neurons appear to become target- dependent at E8[37, 38].

To study trophic dependency of CG neurons during the cell death phase, I sought to demonstrate the requirement of CG neurons for chCNTF *in vivo*. Demonstrating that altering endogenous levels of chCNTF affects the number of neurons that survive the cell death phase is critical to establishing chCNTF as a target-derived trophic factor. Elevating and lowering endogenous levels are both important and necessary criteria. Demonstrating that blocking a factor will reduce survival is important because it shows the endogenous factor is necessary for CG neurons to survive. Elevating trophic factor levels is important because it not only shows the factor can function *in vivo*, but it suggests the factor is naturally limiting.

The ability to rescue 48% more neurons through E14 by overexpressing chCNTF in chick embryos is very significant. From the perspective of rescuing additional neurons by making available a larger target field[8, 11], the magnitude of the rescue from chCNTF alone is at least as great. Considering that in a number of ganglia the majority of CG neurons were spared, and in one case, a complete rescue was seen, suggests that chCNTF may be *the* limiting factor in CG survival. Expression of chCNTF more consistently at higher levels could may help confirm if this is the case.

In contrast to overexpression of chCNTF by RCAS, injection of recombinant chCNTF was unable to rescue CG neurons from cell death. This is in agreement with results published by Oppenheim using purified recombinant bFGF and human CNTF[100, 129], both of which are trophic for CG neurons *in vitro* [53, 86, 136]. Injection experiments have the advantage that one has some control over when and for how long the factor is elevated, and being able to restrict it to particular locations. Unfortunately, since CG survival was only significantly affected under lesion conditions, one cannot take advantage of these capabilities. It has yet to be established if the lack of an effect was really due to insufficient levels of the factor actually reaching the target. CG neurons do respond to rchCNTF *in vivo* under lesion conditions and since elevation of the factor through RCAS does affect CG survival, the inability to rescue additional neurons via injections of recombinant factor is not due to CG neurons being insensitive to chCNTF *in vivo*. If injection approaches are to be used in the future, a better delivery system needs to be used, and some quantitative measure of how much factor actually reached the target tissues should be performed. Additional injection experiments would be desirable because they could help address questions of local influences versus target-mediated effects, as well as possibly defining the trophic dependency of CG neurons *in vivo* at various stages during the cell death phase.

The results from the antisense experiments were unable to demonstrate a statistical difference in CG survival in those embryos infected with RCASBP(A)-antisense chCNTF and those with the virus alone. However, before the conclusion can be drawn that endogenous chCNTF is unable to regulate neuronal survival *in vivo*, a number of technical considerations need to be addressed. First, it was difficult to obtain a large number of CG to analyze from antisense-expressing embryos because the viability was typically about half that of controls, which was not true for the chCNTF overexpression experiments. If chCNTF is important for some critical developmental processes, then this may explain why fewer embryos may have survived. Those that did survive for the duration of the

experiment might also represent embryos with the least amount of antisense expression. Even in those embryos that did survive, there was a greater occurrence when the ganglion could not be found. If a large amount of cell death occurred during the first part of the cell death phase, then the resulting E14 ganglion may be smaller and more difficult to identify. Second, while overexpression of chCNTF did not necessarily need to be exclusively localized to CG target cells, an antisense approach does require infection of the majority of these cells in order to get an effective block. This would be especially true for ciliary neurons that form synapses on striated muscle, where chCNTF receptors will be in very close proximity to the site of secretion. Third, antisense chCNTF experiments may have failed to yield a significant difference due to the inability to obtain a more complete block. Though on average expression of the antisense construct in embryos resulted in a 53% reduction in biological activity in E14 eyes, this may simply be too small of a change for CG neurons to be affected. For example, the effect of a chCNTF *in vitro* is only linear with a narrow range around the ED_{50} of the factor, and a reduction of the factor by 50% does not necessarily result in a reduction in neuronal survival by 50%. Finally, in order to truly correlate reductions in chCNTF levels via antisense expression with reductions in CG neuronal survival, it would be better to screen each target to be sure pronounced infection of the virus in the target tissue was obtained and to only examine the individual CG in those cases where expression was known to be high.

The difficulties encountered in generating a reagent that clearly reduces the amount of factor made available to the neurons should not be a discouragement for future experiments. Now that residues in rat CNTF have been identified as important in receptor binding, it may be possible to generate antibodies to these epitopes that may have potent blocking ability. It might also prove worthwhile to generate additional monoclonal antibodies, this time screening specifically for antibodies that block neuronal survival *in vitro* instead of screening antibodies for their ability to detect chCNTF on Western blots or for specific immunolocalization. The fact that in a couple of experiments there were

fewer neurons in antisense-expressing embryos compared to controls, suggest that the partial block of endogenous chCNTF may have been effective. If additional experiments are done on a larger scale and some of the concerns outlined above are addressed, it may be quite possible to demonstrate a statistically significant effect.

6.3. Do GPA (chick CNTF) serve different functions in the rat and chick?

(Note: this section was modified from an article published in Perspectives on Developmental Neurobiology, vol , 1996).

Similarities between CNTF and GPA. There is considerable overlap in the biological activities of GPA and CNTF. Both were isolated by their abilities to support survival of CG neurons *in vitro* and were subsequently shown to support the survival of a wide variety of neuronal cell types- motor, sensory, and sympathetic. In addition, both CNTF and GPA induce choline acetyltransferase and the same neuropeptides in neonatal rat sympathetic neurons (Fann, 1994). Both CNTF and GPA have also been shown to induce VIP in embryonic chick sympathetic neurons[94]. Finally, the immunostaining pattern of GPA in chicken sciatic nerve is strikingly similar to that of CNTF in rat sciatic nerve [120].

GPA and CNTF also share a number of similarities at the molecular level (Table 1; Fig. 2). The predicted amino acid sequence deduced from the full length cDNAs predict a molecular weight of 21.3 kDa (195 amino acid residues) with a pI of 5.1 for GPA, whereas rat CNTF has a molecular weight of 22.8 kDa (200 amino acid residues) with a pI of 5.7. GPA and the CNTFs all lack an N-terminal signal sequence, yet they are surprisingly hydrophobic. The genomic structure of chick GPA (Nishi, unpublished data) is remarkably similar to that of human CNTF[142] in that both consist of two exons with an

Table 1: Comparison of Ciliary Neurotrophic Factor and Growth Promoting Activity

Property	CNTF	GPA (chick CNTF)
Molecular weight		
Predicted	22.8	21.3
Observed	20.4, 22, 22-23, 24, 25, 28	21.5
Isoelectric point		
Predicted	5.7	5.1
Observed	5.8	4.8
Fold purification required	35,000(sciatic nerve)	80,000(sciatic nerve) 100,000(eye)
N-terminal signal sequence present?	No	No
Internal signal sequence present?	?	Likely
Secreted?	No, or very poorly	Yes, but 50% remains inside the cell

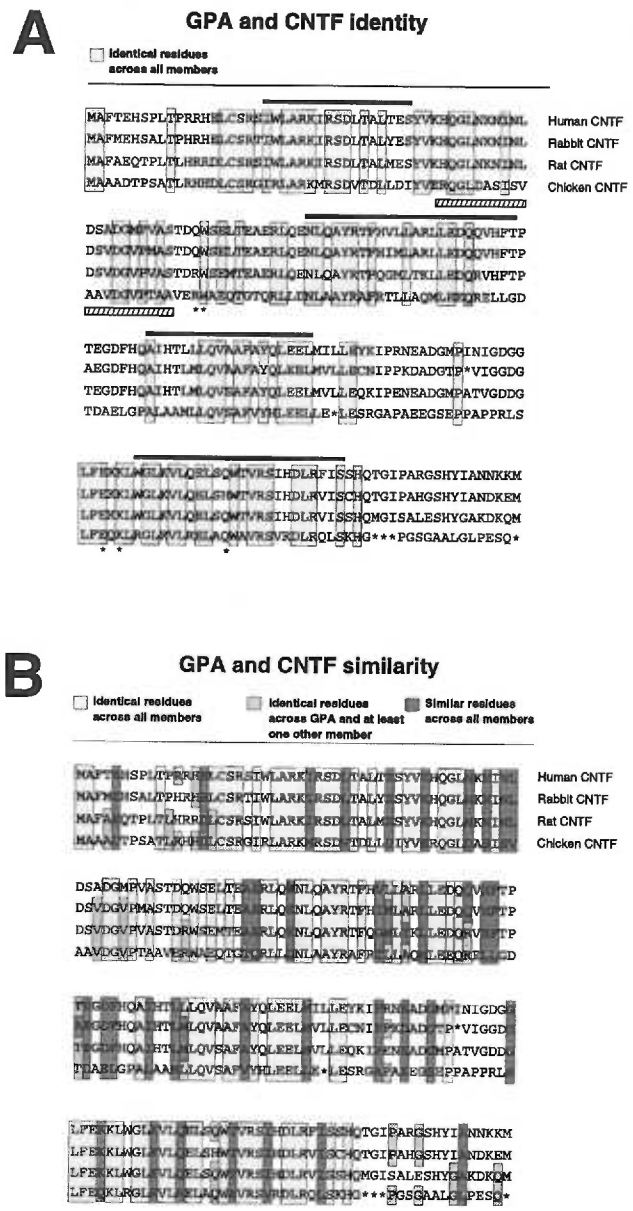


Figure 2. **Sequence alignment of growth promoting activity(GPA) and the three mammalian ciliary neurotrophic factors(CNTFs).** A, sequences were aligned taking into account substitutions and deletions to optimize alignment. Identical amino acids among all four species are boxed in gray. Those residues thought to be important for CNTF biological activity and receptor binding are denoted by asterisks under the four sequences. Portions of the proteins thought to be involved in alpha-helix formation are indicated by a black bar above the sequences. The area in GPA thought to code for an internal signal sequence is indicated by a hatched bar. B, sequence homology increases when conserved residues are taken into account. Amino acids shared by at least one CNTF member and GPA are boxed in medium gray. Conserved amnio acids (like amino acids of the same subcategory, such as small polar for any other small polar) across all four proteins are boxed in dark gray.

intron interrupting the protein coding region of the gene after the 38th amino acid residue (Fig. 3).

Perhaps the most compelling evidence for similarity is in the three dimensional structures of GPA and CNTF. Computer modeling of the predicted structures of CNTF, IL-6 and LIF suggested that the overall folding pattern of these molecules was very similar- all three are comprised of four alpha helical cores connected by more linear linker regions [142]. Subsequent analysis of the crystal structures of CNTF, and LIF[143] confirmed the predictions generated by the computer analyses. Although the crystal structure of GPA has yet to be resolved, computer analysis predicts that GPA contains alpha helices in the same amino acid residues as CNTF (Fig. 2)[144]. As a consequence of the three dimensional structural similarities, this class of molecules has been called the neuropoietic cytokines[142] and is now known to include CNTF, GPA, IL-6, LIF, human growth hormone[145], oncostatin M (OSM), granulocyte colony stimulating factor (GSCF)[146], and cardiotrophin-1[147].

In addition to the three-dimensional structural similarities, GPA and CNTF signal through a commonly organized receptor complex (Table 2). The polypeptide responsible for conferring specificity with respect to ligand binding for CNTF is the alpha subunit, termed CNTFRα, which was isolated by affinity chromatography from a human neuroblastoma cell line[117, 148]. More recently, GPARα was cloned by using a rat CNTFRα probe to screen a chick sympathetic neuron cDNA library[95]. GPARα expression was detected on chick ciliary ganglion neurons, suggesting that it is likely to be mediating the effects of both CNTF and GPA. Both Rα subunits encode an extracellular molecule attached to the membrane by a phosphoinositide linkage and both Rα subunits have been shown to transduce a signal by complexing with two other polypeptides:

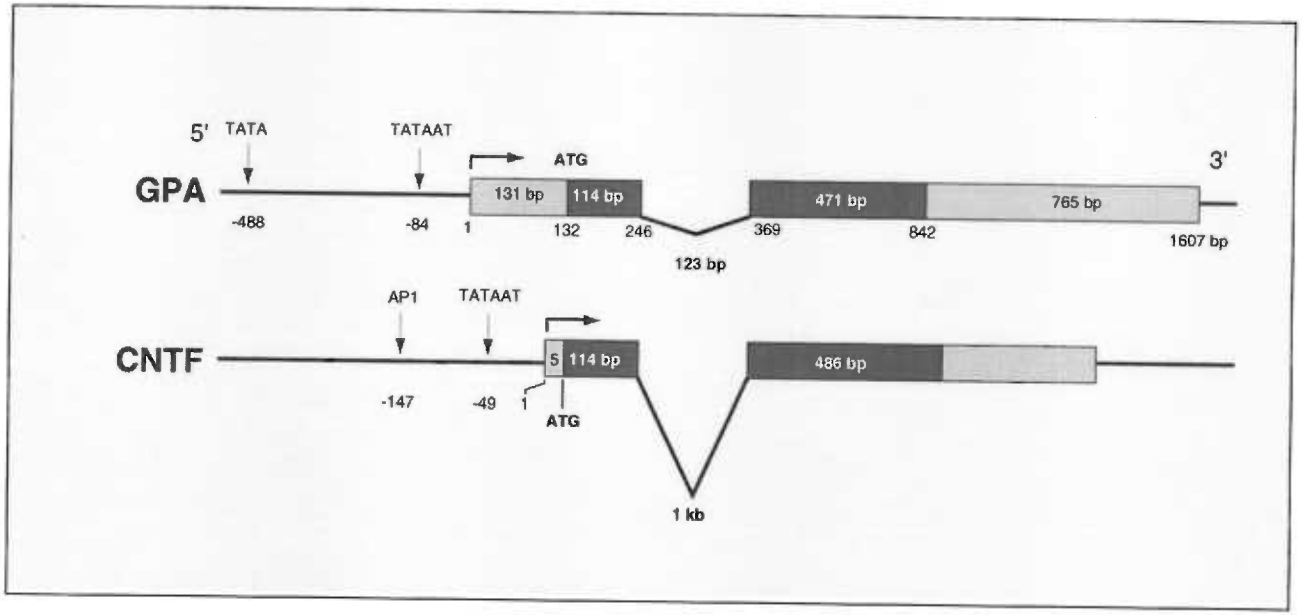


Figure 3. **Genomic structure of chick growth promoting activity (GPA) and human ciliary neurotrophic factor(CNTF).** Gray portions denote noncoding regions of the message, white areas protein coding regions. Transcriptional start sites are indicated by bent arrows. Although the size of the intron varies, in both chick GPA and human CNTF the intron begins at amino acid 38. Note: introns are not drawn to scale.

leukemia inhibitory factor receptor beta (LIFRβ) and GP130 to activate a soluble tyrosine kinase inside the cell[95, 96, 149, 150].

Differences between CNTF and GPA. The primary structure of GPA differs more from rat, rabbit, and human CNTF than that seen across other neurotrophic molecules such as the neurotrophins. The CNTFs share 76% amino acid identity with each other (Fig. 2), while 39% of the amino acids of GPA are identical with the three CNTFs. When one takes into account conservative amino acid substitutions, the similarity of the CNTFs to one another increases to 89% while the similarity between GPA and the CNTFs is 61%. In contrast, there is approximately 85% identity between chick and mouse NGF[151] or >90% identity between chick and mouse acidic fibroblast growth factor[152]. The differences in structure between GPA and CNTF are significant enough that CNTF cDNA probes are unable to hybridize to GPA-containing plasmid DNA even at extremely low stringency (Nishi, unpublished observations) and none of the GPA- recognizing antibodies we have isolated to date are able to recognize even microgram quantities of pure recombinant rat CNTF[120].

Since GPA and CNTF fit by virtue of their folding patterns into the neuropoietic cytokine family, one should compare the homology across species to that seen in other members of the family. Here, the results are mixed. Interleukin-6 (IL-6) differs considerably from mouse to human with only 46% amino acid identity. This is surprising in light of the ability of IL-6 from one species to produce biological effects in cells of others species, but consistent with the differences seen between GPA and CNTF. On the other hand, mouse LIF, whose biological activity is highly species-specific, is approximately 90% identical to human LIF.

A more significant difference between GPA and CNTF is that GPA is secreted considerably more efficiently than CNTF. Transient transfection experiments initially performed with CNTF alone indicated that biological activity could be recovered from extracts of cells but not from the cell culture medium[54]. When GPA was cloned, GPA and CNTF constructs in the same expression vectors were transfected into sister cultures of cells and neurotrophic activity was detected in GPA transfected cell medium but not in the medium of CNTF transfected cells[54]. Controls performed by monitoring lactate dehydrogenase released from cells demonstrated that the trophic activity in GPA transfected cell medium could not be accounted for by cell lysis. One caveat to the transfection studies is that secretion was established by bioassay, thus if the CNTF were released but more rapidly inactivated, then CNTF secretion would not be detected. However, more recent studies performed in our lab using ^{35}S-methionine labelling and immunoprecipitation of CNTF from transiently transfected COS cells do not detect CNTF in the medium (Reiness and Nishi, unpublished data).

We have postulated that an extra hydrophobic domain at amino acids 40-60 found in GPA but not in CNTF is responsible for directing the secretion of GPA[54] (Fig. 4, Appendix, Section 7.4). In order to test this hypothesis, we have constructed a chimeric molecule containing the first 58 amino acids of GPA and the last 152 amino acids of CNTF. This chimeric molecule is secreted and is biologically active[54]. This difference in biological availability suggests that GPA and CNTF may serve considerably different functions. If these differences in function are species-specific, then this is highly unusual and not found in other cytokine families; for example, across species the neurotrophins are all constitutively secreted molecules.

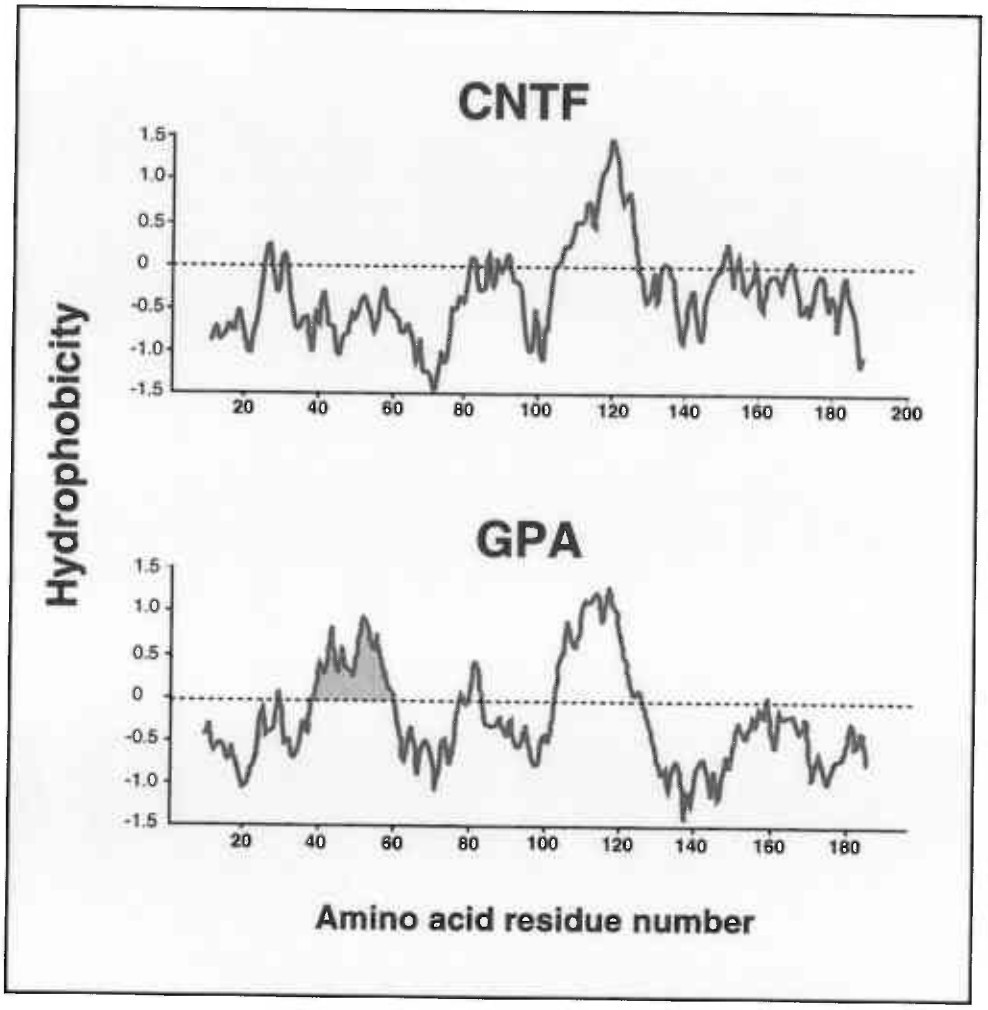


Figure 4. **Hydrophobicity plots comparing chick growth promoting activity (GPA) and rat ciliary neurotrophic factor (CNTF).** Hydrophobic regions are indicated as positive values, hydrophilic regions of the proteins as negative values. GPA has a second region of hydrophobicity (hatched area) that is not present in CNTF and may act as a secretion signal.

Another intriguing difference between GPA and CNTF is in their interaction with binding sites on the surface of chick sympathetic neurons[94]. ^{125}I-CNTF was used to demonstrate saturable binding to a high affinity site on embryonic lumbar sympathetic neurons. This binding was competed for with unlabeled CNTF, as expected for a specific site. However, unlabeled GPA was at least 10-fold less effective at competing CNTF binding than CNTF, even though the unlabeled molecules were equipotent in supporting CG neuron survival. These differences in binding cannot be attributed to species differences since the rat ligand had an apparently greater binding efficiency to the chick receptor. Interestingly, all the amino acids identified as contributing to receptor binding or biological activity of rat CNTF[157] are also identical with those found in chicken GPA (Fig. 2). Additional residues may be necessary to confer the differences in binding of GPA and CNTF to the chicken receptor. Preliminary work in our lab suggests that CNTF has a lower ED_{50} for supporting survival of sympathetic and sensory neurons than GPA; whereas, GPA has a lower ED_{50} than CNTF on CG neurons. Among many other possibilities (such as differences in intracellular signal transduction), these observations nonetheless lead one to wonder whether there are two different receptors in one species of animal that have different affinities for GPA and CNTF.

Do GPA and CNTF serve different functions? If GPA is chicken CNTF, then they should serve parallel functions in their respective species. To date, most of the descriptive information regarding the similarities in biological activities of CNTF and GPA have been obtained from cell culture studies and few studies have been performed *in vivo*. CNTF has been proposed as a lesion-induced repair factor in the peripheral nerve because extremely high quantities of CNTF protein (25,000 TU/mg [50]) has been detected in intact nerves. If

the stored CNTF cannot be secreted, the only mechanism for its release would be by cellular injury. On the other hand, GPA is 25 times less abundant in chicken sciatic nerve (970 TU/mg[53]) and the constitutive secretion of GPA may suggest a different functional role.

Although we have established that GPA meets a number of criteria for being a true target-derived trophic factor for avian CG neurons, it is not known whether CNTF serves the same function in the mammal. GPA supports survival of CG neurons in culture, is expressed in target tissues of CG neurons during and after CG neuron cell death, and can be secreted by cells that synthesize it[54, 120]. Hence, GPA appears to be a true ciliary neurotrophic factor in the chicken. In contrast, the development of the rodent CG is less well understood. For example, whether the rodent CG undergoes cell death has not been investigated, perhaps because the rodent CG is very small (ca. 300 neurons) and distributed over the oculomotor nerve[158], unlike the ciliary ganglion of the bird[35] or of primates[33] which are larger (1200 in the human and 3200 in the chicken) and more organized in their structure. Correspondingly little is known about the expression of CNTF during embryonic rodent development, especially within the eye. Finally, although mice that lack the gene for CNTF have been produced[159], it is not known whether the number of CG neurons is correspondingly reduced, or even whether the CNTF knockout mice lack or have a significantly reduced pupillary response to light.

Although evidence does not support CNTF as a target-derived trophic factor for mammalian motor neurons, GPA remains a candidate for a target-derived motor neuron trophic factor. GPA supports the survival of chick motor neurons in culture (M. Sendtner, personal communication) and GPA supplied to the chicken embryo is as effective as CNTF in rescuing spinal cord motor neurons from cell death (R. Oppenheim, personal

communication). GPARα is also expressed by embryonic motor neurons in the spinal cord and, since GPA can be secreted from cells, there is a mechanism for making GPA available extracellularly. Although CNTF is not expressed in embryonic rat muscle, the expression of high levels of GPA in embryonic chick iris and ciliary muscles is encouraging; however, it is not known whether embryonic chick skeletal muscle expresses detectable GPA mRNA or protein.

Finally, the widespread distribution of CNTFRα expression and the limited expression of CNTF suggests that related molecules may interact with the CNTF receptor. For example, CNTFRα mRNA is expressed throughout the central nervous system and in the tissues of the rat, however, CNTF mRNA is limited to the olfactory bulb, hippocampus, cerebellum, and sciatic nerve[70]. Recently, CNTFRα knockout mice were reported to have a significantly more severe phenotype than the CNTF knockouts[160] dying shortly after birth, also suggesting that other known neuropoietic cytokines or novel CNTF-like molecules normally interact with CNTFRα during development.

Summary. The similarities in primary, secondary, and tertiary structure together with similarities in the receptor complexes through which these ligands act, place GPA and CNTF into the neuropoietic cytokine superfamily. Differences in the abilities of GPA and CNTF to be secreted as well as differences in the binding affinities with which these molecules interact with sites on the surfaces of chicken sympathetic neurons have been noted. Together with differences between the distribution of CNTF receptors and their ligands and the significantly greater effects observed when the CNTFRα versus CNTF genes are knocked out suggest that a CNTF subgroup of neuropoietic cytokines may exist. This would be analogous to subgroups that have already been identified in the TGFβ

superfamily of cytokines. Clearly, this issue will not be resolved until a more CNTF-like molecule is isolated from the chick or a more GPA-like molecule is isolated in a mammal. If a subfamily of factors exists, then CNTF-like molecules might be discovered with important therapeutic applications.

6.4. What remains to be tested in order to more firmly establish chCNTF as a target-derived factor?

6.4.1 With respect to establishing chCNTF as a true target-derived neurotrophic factor?

As outlined above, evidence for chCNTF playing a role as a target-derived trophic factor for CG neurons falls into two categories, descriptive evidence and experimental manipulations of chCNTF levels *in vivo*. The descriptive evidence is completely consistent with a target-derived trophic factor as outlined from the neurotrophic theory. Overexpression experiments and recombinant chCNTF experiments demonstrate that CG neurons are sensitive to chCNTF *in vivo* and it can enhance CG neuronal survival. These were only partial effects, however, and it remains to be seen if better delivery and expression conditions results in greater levels of survival or if other required aspects of the target, such as additional neurotrophic factors, limit the effects of chCNTF. Experiments designed to lower chCNTF levels through an antisense approach were successful in significantly reducing chCNTF biological activity in the targets, and in showing a small reduction in CG survival in two experiments, but overall failed to conclusively show a definitive reduction in CG neuronal numbers. Changes in the experimental design as outlined in the discussion section of Chapter 4 may help surmount the problems of high variability in neuronal numbers in untreated CG as well as achieving a better infection and spread of the virus to deliver the antisense.

Alternative approaches have already been tried as ways to reduce chCNTF levels by other means. These include inhibitory synthetic peptides (Appendix, section 7.3),

production of blocking antibodies in hens delivered naturally to chick embryos (Appendix, section 7.4), attempts at producing polyclonal blocking antibodies in rabbit and injection into chick eyes, and attempts to make monoclonal blocking antibodies from mice intended to be delivered via the chorioallantoic membrane to chick embryos (not shown). Unfortunately, none of these alternative approaches yielded a reagent that would be potent enough to effectively block *in vivo*. Strategies that were not tried but may yield successful results include the production of a dominant negative receptor expressed by RCAS, or injection of a soluble receptor that would compete for ligand binding but not become part of the receptor complex. Alternatively, a sequence specific cleavage of chCNTF message through targeted ribozymes could be used. This relatively new approach relies on using RCAS to deliver a transgene expressing an engineered tRNA with a ribozyme encoded in it specific for a given message.

6.4.2 With respect to neuronal cell death in general? The vast majority of what we know about neurotrophic factors and how they function in general is based largely upon a single family of factors- the neurotrophins. The action of NGF on sympathetic neurons remains the best example of a target-derived trophic factor that regulates neuronal cell death. Whether or not all target-derived trophic molecules work the same, or whether trophic dependency is equivalent in other neuronal populations remains to be seen. The relative simplicity of the ciliary ganglion makes it very useful in addressing questions about *how* neurotrophic factors regulate neuronal cell death.

Based on work by other groups and the findings in this study a composite graph correlating the number of CG neurons during the cell death phase overlaid with chCNTF trophic factor levels can be made (Fig. 5). This graph is speculative, since the actual position of trophic levels cannot be assigned a particular region on the graph. If chCNTF functions the way the neurotrophic hypothesis specifies how such a factor might work, one could make the following predictions. At the start of cell death CG neurons are very

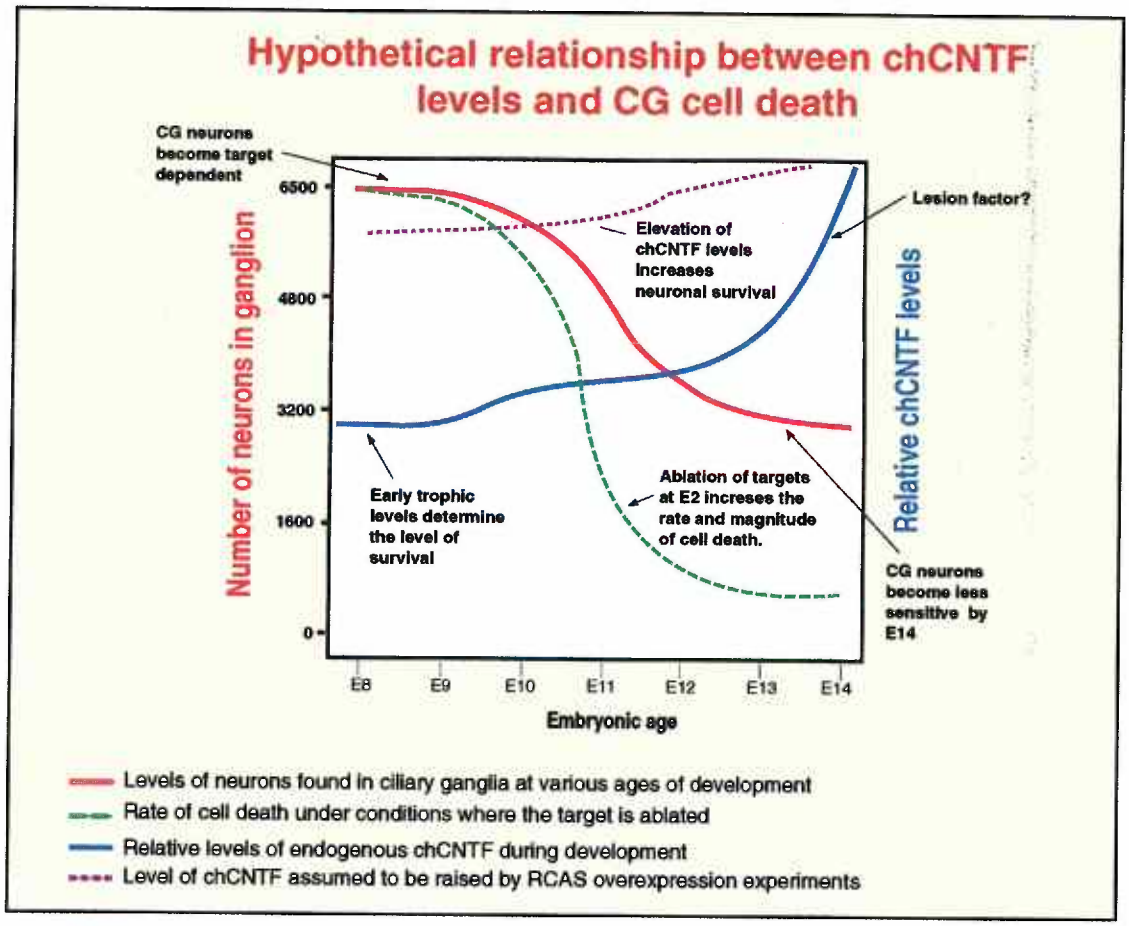


Figure 5. Composite graph showing hypothetic relationship between the shape of the CG cell death curve and chCNTF levels. Published values from Landmessar and Pilar, 1974 for CG neuronal counts during development (shown in red) are superimposed with the relative levels in chCNTF protein as determined by immunocytochemsitry and quantitative bioassays (blue line). Although the absolute level of the factor is not known, assuming it is limiting and functioning as would be predicted by the neurotrophic hypothesis, the relationship between chCNTF levels and the number of neurons still present in the ganglion might look as depicted above. From work performed as part of this thesis and observations made by other investigators, it is likely that the key period in determining how many CG neurons ultimately survive is decide in the first few days of cell death. As the embryo progresses further into the cell death phase, CG neurons become less dependent upon the factor and trophic levels rise to ensure plenty of chCNTF exists for those neurons that have successfully competed thus far. If one artifically elevates chCNTF levels, more neurons would be capable of competing for the additional factor and more CG neurons will survive.

sensitive, perhaps the most sensitive they will be during this phase, to trophic influences by the target. Before cell death phase begins, CG neurons already have receptors for chCNTF and at the start of cell death, all the neurons have become chCNTF dependent. At E8, chCNTF is present, but its levels are kept low enough such that insufficient trophic molecules are available to keep all CG neurons alive. Each neuron would compete for the available factor, perhaps by adjusting the number of synapses it makes on the targets. Removal of the target before the cell death phase not only results in most of the neurons dying off once cell death has started, but the rate of cell death occurs much faster. If one artificially elevates the early chCNTF levels it will allow some of the neurons that would have otherwise died off to successfully compete for the additional factor. As this developmental phase continues, CG neurons become less dependent upon the factor, but are still target-dependent. The lower dependence on the factor is not due to a loss of the receptors as CG neurons continue to synthesize them long after the cell death phase is complete. ChCNTF levels rise during the latter half of this time window to supply ample factor to those neurons that have successfully competed thus far. Some of the rise in trophic levels is due to the presence of more target cells being made as the target matures. By the end of the cell death phase, the neurons are largely insensitive to the factor, but the target continues to synthesize the protein, perhaps to serve other functions later in development.

In studying target dependency of neurons one also has to consider target-supplied influences other than neurotrophic factors. The possible involvement of other, non-trophic properties of the target in regulating cell death has already been shown through regulation of synaptic activity. Although the results are not completely consistent, blocking of synaptic activity *in vivo* through agents such as curare or alpha-bungarotoxin during the cell death phase results in nearly twice the number of neurons at E14, or in these studies about 90% of E8 levels[85]. Similar results were shown in the spinal cord[161]. This does not mean that synaptic activity would have to work through mechanisms

exclusive of neurotrophic factors. Synaptic activity might, for example, regulate the production or release of trophic factors.

It is somewhat puzzling how neurons can be matched to their targets if the target is going through so many changes itself. For example, the observation that smooth muscle actin, and likely the number of smooth muscle cells as well, increases so dramatically during the time when neurons become dependent upon their target for survival is surprising. At a time when significant numbers of neurons have already begun to die (E10), the target contains relatively little smooth muscle. In the iris, a transition from smooth to striated muscle occurs, again, during this same time window of development. While these changes may explain why chCNTF levels increase during the cell death phase, it does not explain how chCNTF is kept limited.

6.5. Conclusions.

ChCNTF meets most of the requirements for a target-derived trophic factor, and obtaining the final pieces of evidence to sufficiently establish it as being such a molecule are close at hand. Once this information is known, it paves the way for asking questions about how trophic factors actually regulate cell death in the CG. Ironically, despite an enormous literature on the identification and characterization of novel trophic factors and the neurons they may act upon, relatively few of the questions posed at the beginning of this century have been answered. Perhaps when some of these questions are applied at a molecular level to the relatively simple system of the CG, some important clues may fall out of this research.

Appendix

7.1. *In vitro* trophic dependency of E14 and E16 CG neurons vs. E8 neurons. While it was clear that purified rchCNTF could promote survival of CG neurons *in vitro*, the trophic dependency of CG neurons from older embryos for this factor was unknown. ChCNTF message, protein, and total units of biological activity increases substantially by E14, which might suggest that the neurons have a greater requirement for the factor. However, Alcain showed that the further along during the CG cell death phase one examined, the less dependent CG neurons became upon eye tissue conditioned medium[162]. Since chCNTF is a likely candidate for a target-derived factor and could be a major component of conditioned medium from cells of the eye, I questioned whether CG neurons remained dependent upon chCNTF at the end of the cell death phase. Even though trophic levels in the target are higher at E14 than E8, E14 and E16 CG neurons seemed very unresponsive to chCNTF. Even at concentrations as high as 50 ng/ml, only a very subtle difference could be seen between E14 and E16 neurons grown with or without any added factor. E14 or E16 CG neurons survived, appeared healthy, and extended neurites in the absence of any added chCNTF. It should be noted, however, at E8, CG neurons dissociate well and most neurons survive the mechanical and trypsinization procedure. E12 and older ganglia, however, are more difficult to dissociate and the percent survival of E14 neurons under the best circumstances was only 50% of that of E8 ganglia (even taking into account the fewer neurons that are naturally present in older ganglia). Collagenase had to be in the dissociation procedure in order to get reasonable levels of survival.

It was possible that the decreased sensitivity to rchCNTF could be due to a much higher level of non-neuronal cells present in E14 and E16 cultures. Attempts to eliminate non-neuronal cells from the cultures through ficoll gradients, pre-plating on non-coated plates, and inclusion of mitotic inhibitors were only partially successful. Even after this enrichment, no significant difference could be seen. When grown as single neuron cultures

by limiting dilution into Terasaki plates, CG neurons from older age ganglia were still found to be largely independent of factor. Such neurons continued to extend neurites (though at much lower levels than if eye extract was added), and cell bodies were large and phase bright. E14 neurons show a range of cell diameter, with some neurons having more than 4 times the area of E8 neurons, as would be predicted from histological studies[35].

7.2. Determination of the rate and level of cell death in the chick CG: a comparison with published values. The ciliary ganglion is a very well studied and characterized system. Numerous histological studies have been performed on human[33], rabbit[34, 163], rat[164]), chick[35], pigeon[165],cat CG[36], and dog[166] CGs. Studies have been performed at both the light and electron microscopy level. In the chick CG, the level of cell death has been well documented by studies performed by Landmesser and Pilar, 1974[37, 38]. In this study, the number of neurons was determined at various embryonic ages beginning at E7, the start of cell death, through E20, six days after the cell death phase is largely over. This study determined that roughly 6500 neurons are present at E8 and about 3200 neurons at E14, thus resulting in approximately 50% of the neurons being eliminated by developmental cell death. In a separate study, however, Furber found beginning at E6, the total number of neurons to be 6000, and the level of cell death to be about 63% [137]. In CG cell culture studies, I have on a number of occasions calculated the presence of approximately 14,000 neurons in an E8 CG. These differences could be due to species differences in the chick embryos being examined. If that is the case, then it is important to reproduce a cell death curve for the particular species of chicken being routinely used. If neuronal cell death is shifted by a day or two or if the levels of cell death are greater than that of published values, then it may have important ramifications of the design of future experiments and possibly the interpretation of results.

To perform these studies, chick embryos were incubated the appropriate number of days and staged according to the Hamburger and Hamilton staging series[118], and their CGs removed and placed in fixative. CG neurons were processed, sectioned, and stained as outlined in Chapter 2. E8, E9, and E10 ganglia were counted at a total magnification of 600X, counting every fifth section in a series. E11 ganglia and older were counted at 400X with every tenth section being counted. The number of profiles was converted to total number of neurons by dividing by the number of sections counted and multiplying by total number of sections in the series. Embryos of white leghorn chickens obtained from Oregon State University Poultry Science showed many more neurons present in the ganglion than estimated by those of Landmessar or Furber (Fig. 1). While the overall shape of the curve was essentially the same as that reported by Landmessar[37], the extent of cell death was more pronounced (72% versus 50%).

7.3. Preparation of blocking antibodies in adult hens. Systemic administration of blocking antibodies to NGF in rats was fundamental in establishing NGF as a target-derived trophic factor for sympathetic neurons. Attempts to produce blocking antibodies chCNTF, however, was largely unsuccessful. Of 4 monoclonal antibodies to chCNTF screened, none had any blocking activity, either alone or in combination, on CG neurons grown in the presence of rchCNTF. Rabbit antisera to chCNTF did have some blocking ability, but even at high concentrations of the antibody (1:400) in culture, only a 60% reduction in CG survival was seen *in vitro*. For an antibody to work effectively *in vivo*, one would need blocking ability at lower concentrations with greater efficacy *in vitro*. Experiments where rabbit anti-chCNTF was injected into embryonic chick eyes were performed on shell-less embryos, but counts were never made from the ganglia because the outcome of these experiments was likely to be inconclusive.

The use of large amounts of antisera was not feasible for these experiments and an alternate approach was tried: the use of adult hens to produce blocking IgY antibodies to

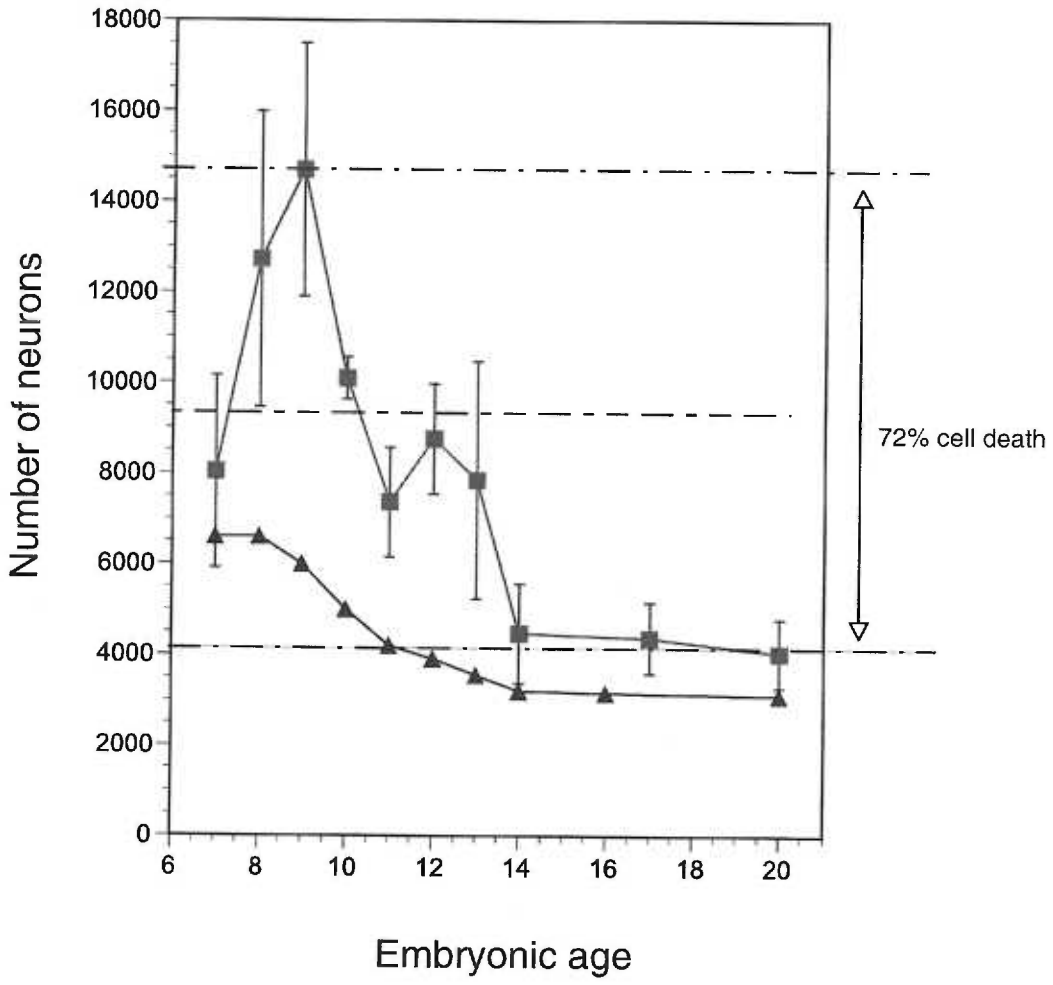


Figure 1. Comparison of CG neuronal cell death in White Leghorn Chickens from the OSU Poultry Science Department from that of published values. The number of neurons present in each ciliary ganglion from embryos at various points through the cell death phase was calculated from profile counts of thionin-stained 8 micron paraffin sections. Estimates were made based on the counting method of Landmessar and Pilar, 1974, and compared accordingly. (■—■ This study, ▲—▲ Landmessar and Pilar, 1974). The maximum number of neurons and the extent of cell death is significantly different beween the two different studies, but the rate of cell death is approximately the same. Error bars represent standard errors of the means.

chCNTF. The rationale for the experiments is as follows: if the hens could make an immune response to chCNTF, then the possibility exists that endogenous chCNTF could be blocked in the embryos, since egg yolk contains high levels of IgY (IgG-like) antibodies passed on from the mother. ChCNTF was crosslinked either to itself or to KLH and injected into 2 adult hens. Both hens were found to make an immune response to chCNTF. Diluted crude yolk from these hens could be used directly on dot blots, Western blots, and ELISAs against the purified molecule, or bacterial cell extracts expressing chCNTF (see Fig. 2). Both the crude yolk and IgY partially purified from whole yolk was tested for blocking ability to block neuronal survival in CG cultures supplemented with chCNTF. Only one of the hens was found to make antibodies with any level of blocking activity. Unfortunately, blocking ability was only detected at dilutions of 1:40 or lower, and therefore not likely to effect endogenous chCNTF levels *in vivo*. Ganglia from E14 embryos taken from the eggs of this hen showed no difference in CG neuronal profiles between the three, though only one ganglion was counted for each of the two hens and an unimmunized hen.

In summary, of mouse monoclonal, rabbit polyclonal, and chick polyclonal antibodies, one was found to have sufficient blocking activity against chCNTF *in vitro* and chickens making weakly blocking antibodies to chCNTF had no effect on CG survival *in vitro*. The fact that hens can make an immune response to chCNTF, some blocking activity was detected, and high levels of anti-chCNTF IgY antibodies were observed makes this a worthwhile system that could possibly be used for further studies, perhaps against peptide fragments of rchCNTF.

7.4. Synthetic peptides. Given the problems with aggregation of chCNTF at high concentrations and the problems of diffusion of large molecules through tissues, it would be desirable if a small peptide could be found that would mimic chCNTF activity. Furthermore, if a peptide could be made that has inhibitory activity on CG cultures, then

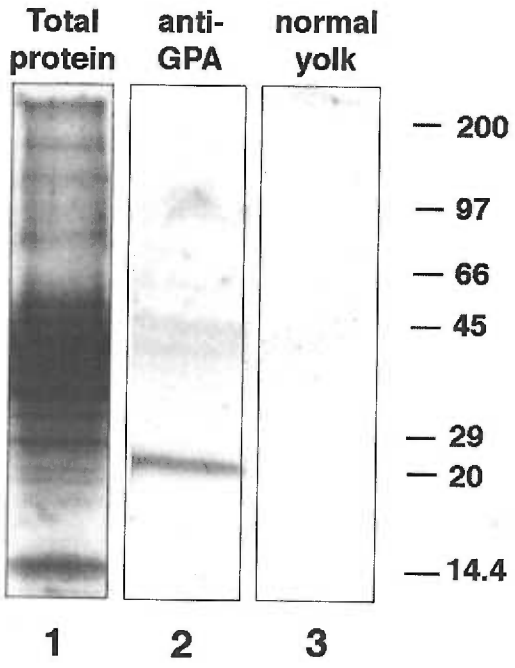


Figure 2. **Specificity of chicken anti-chCNTF antibodies.** Diluted crude yolk from eggs from a hen immunized with chCNTF was tested for its abililty to specifically recognize chCNTF antigen on a western blot. Equal amounts of a crude bacterial lysate from a strain of *E. coli* expressing chCNTF are present in each lane. Lane 1 shows a coomassie stained portion of an equivalent lane. A 21 Kd band was detected by anti-chCNTF yolk (lane 2), but not by a control yolk from a un-immunized hen (lane 3). The lighter bands detected by anti-chCNTF yolk in the 40-45 Kd region may corespond to chCNTF dimers that are often present when recombinant chCNTF is separated by SDS-PAGE.

the possibility exists that such a peptide could be used as a competitive inhibitor for endogenous chCNTF given that endogenous levels are low and high concentrations of synthetic peptides could be made. By aligning chCNTF (GPA) with other CNTF sequences from other species, four regions of homology can be found. These regions probably correspond to conserved alpha helical structure present in this class of molecules (see Discussion, Section 6.3). Of these four regions, two were chosen and synthetic peptides made as part of a collaboration with Genentech. At the time these were chosen, very little was known about CNTF in general and the crystal structure for LIF and human CNTF were not known. Since then, this information has been added and site-directed mutagenesis sites performed on rat CNTF to assess which residues are important for binding [157] (See Fig. 3).

In testing these peptides on CG neuronal cultures, I found peptide #1 to have CNTF-like biological activity and peptide 2 to have partial blocking ability. Unfortunately, these effects were only seen when used at micromolar concentrations. Considering the cost of producing such peptides on a large scale and the relatively low solubility of these peptides in aqueous solutions, it was decided not to proceed with this line of research.

7.5. The use of shell-less chick embryo cultures for injection and application of factors and reagents *in vivo*. For more than 40 years, researchers have taken advantage of the fact that chick embryos can be grown outside the shell. A wide range of supports have been tested for their ability to maintain embryo viability and normal growth and development. One of the most successful techniques documented has been the tripod-plastic sling method of Dunn [122]. Using this method, I was eventually able to obtain viability rates (at 24 hours) of up to 92% of the eggs originally opened, with an average of around 70% for most of the later batches of cultures. Embryos that were set up at E0 did not develop, but those set up after E1.5 had high success rates, although such embryos are very small and transparent at this point and difficult to see. Embryonic day

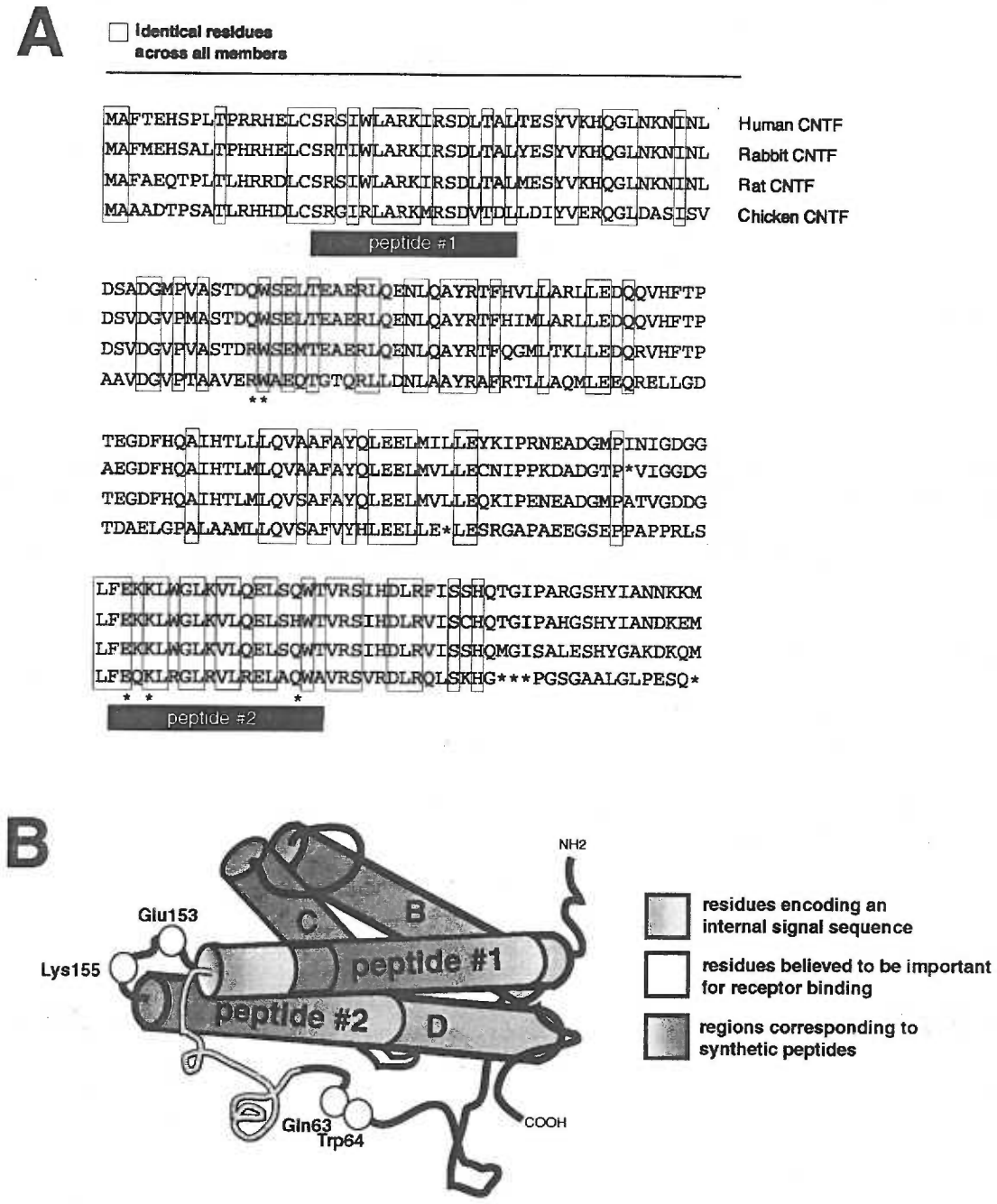


Figure 3. **Location of chCNTF synthetic peptides within the chCNTF molecule.** Two peptides were chosen for synthesis based on regions with the highest degree of conservation between the chick and mammalian forms of CNTF and on predicted solubility in aqueous solution. *A*, sequence alignment of chick CNTF with the mammalian sequences, regions of chCNTF that correspond to the peptides are underlined. *B*, predicted 3-D structure of chCNTF based upon conserved motifs in the known crystal structures for human growth hormone, LIF, IL-6, and human CNTF. Peptide 1 acted as an agonist, and peptide 2 as an antagonist when used at micromolar concentrations on CG neurons *in vitro*.

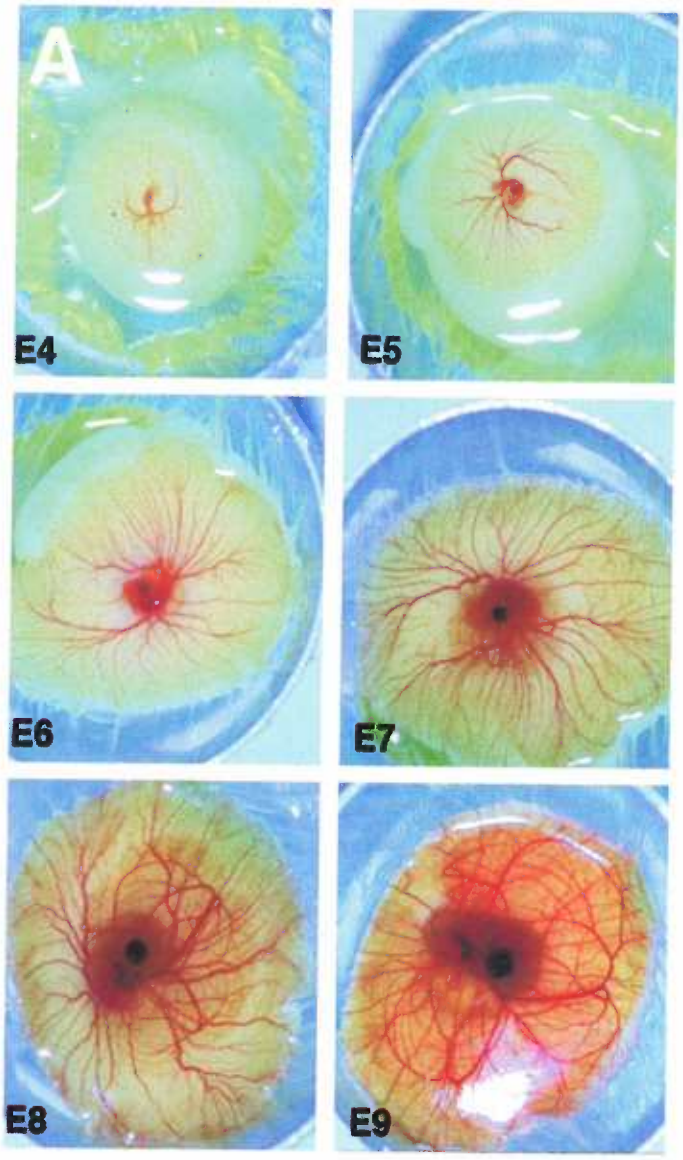
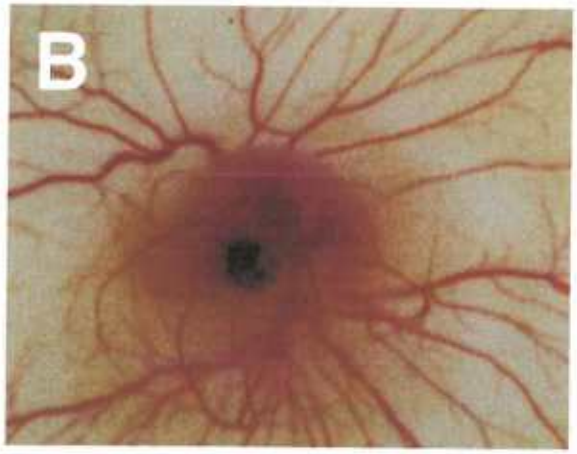
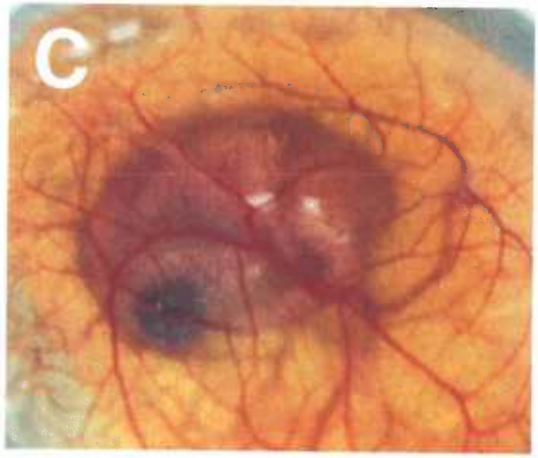


Figure 4. **Optimization of shell-less chicken cultures for *in vivo* experiments.** Embryos placed into sterile shell-less tripods between E2 and E4 survive with good viability and develop normally. Panel *A*, when grown under high humidity conditions, shell-less embryos are indistinguishable from embryos grown in intact eggs. Panel *B*, close-up view of E7 chick embryo as it appears just before injection. Notice the easy accessibility of the eye for injections. Panel *C*, E12 shell-less chick embryo-notice the targets are easily accessible even at this age. Panel *D*, since embryos always float to the top when grown in shell-less cultures, injections could be made from many angles. Both narrow bore pulled glass pipettes and 27-gauge needles could be used.





2.75 appeared to be the best starting point, yielding the highest viability and the healthiest embryos, while E4.5 was the latest age that such cultures could be set up with good viability ratios. Beyond this time point, most embryos die from hemorrhaging as E5 and older embryos have extensive vasculature. Most embryos that survived the first 24 hours survived quite well through E10 (Fig. 4). After E10, the percent survival decreased slightly and after E14, viability decreased substantially. In an experiment where a small set of cultures were allowed to go to hatching, no embryos actually "hatched", however, 2 embryos survived to E20. Shell-less cultures developed at the same rate as embryos in intact eggs, even though the embryos suffered an initial trauma after being placed in the tripod.

Shell-less cultures proved to be invaluable for injections of RCASBP(A) retrovirus (Chapter 2), recombinant chCNTF (Chapter 3), and for injections of rabbit anti-chCNTF blocking antibodies (not shown). Embryos always floated to the top of the amniotic fluid with the right side of the embryo almost invariably facing up. The presence of the chorioallantoic and amniotic membranes posed little problem as these membranes could be either cut or poked through with needles with no harm to the embryo, as long as an artery was not damaged. While windowed eggs had higher viability rates, the superior accessibility to the embryos made this the method of choice for the majority of the *in vivo* experiments performed.

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